ARTIFICIAL INSEMINATION OF CATTLE

A critical review of the literature

by

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Although artificial insemination of cattle has, within a relatively short space of time, become a firmly established practice, I consider it only fitting to record that those of us engaged in this field are greatly indebted to the late Dr. Arthur Walton for the work he did in the early development of the techniques in this country.

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A. Introduction

Artificial insemination is now widely used in the breeding of cattle and several textbooks have dealt with the subject (Hammond, Edwards, Rowson and Walton, 1947; Perry, Bartlett, Edwards, Terrill, Berliner and Jeffroy, 1947; Rowson, Day and Griffiths, 1948; Herman and Madden, 1950; Millar and Ras, 1952; Laing, 1955; Van Rensburg, 1957). The early work on artificial insemination and on the semen of the domestic animals has been adequately reviewed by Anderson (1945).

The present extent of use of artificial insemination in different countries is shown in Table 1 and this data indicates that further expansion is still possible in many countries. The arguments put forward in 1941 for its trial on a field scale were discussed in restrospect by Walton (1958) who also maintained that the economic benefit to the small farmer and that the wider use of progeny tested bulls would result in the further extension of its use in the future. The nature of the development in the different breeds in England and Wales (see Table 2) is somewhat different from what might have been anticipated; this is chiefly due to the relative decrease in the demands for insemination from bulls of dual-purpose breeds (in particular the Dairy Shorthorn) and to an increase in the demand for crossing with beef bulls.

The application of artificial insemination in cattle has several

advantages. The small farmer has a choice of good bulls of several breeds for less than it would cost him to keep a bull of his own (Hammond, 1953); the 'communal bull' is much less common and it is generally agreed that the almost complete disappearance of trichomoniasis in England and Wales (Report 1958a) is due to this; increased production by the use only of proven bulls and breeding from a bull that may not be able to serve because of illness or injury or that may be too heavy for certain cows is made possible. The possible usefulness of artificial insemination for upgrading indigenous stock in underdeveloped territories has also been accepted, but it cannot always be practical under the conditions existing in many of these countries.

The success of artificial insemination depends very much on the accurate detection of heat by the farmer (Smith, 1958). For this reason a service is of limited value where cattle are not kept under close observation and in areas with poor means of communication.

TABLE 1

EXTENT OF USE OF ARTIFICIAL INSEMINATION (SMITH, 1960)

Country	Number of cows inseminated	Percentage of cow population inseminated
Denmark	1,526,000	98
Netherlands	1,085,000	68
England and Wales	1,982,000	66
Sweden	517,000	38
U.S.A.	6,646,000	30
Irish Republic	483,000	40
Northern Ireland	109,000	36

Western Germany	1,450,000	30
France	4,000,000	40
Scotland	110,000	19
Canada	524,000	10
New Zealand	328,000	16

TABLE 2

DEVELOPMENT OF ARTIFICIAL INSEMINATION IN ENGLAND AND WALES

Type of bull	1948/49	Per cent of total inseminations	1959/60	Per cent of total insemination
Dairy	225,840	58.2	1,190,980	60.1
Dual Purpose	129,181	33.3	185,762	9.4
Beef	32,742	8.5	605,441	30.6
Total inseminations	387,763	-	1,982,133	-
Estimated proportion of cow population inseminated	11.0 per cent		66.0 per cent	

Prophecies that the prolonged use of artificial insemination will, in some way, affect future generations of cattle have as yet received no support. Rae (1958) found that in New Zealand a change to artificial insemination had no effect on sex ratio, incidence of multiple births or barren heifers, while Jaskowski (1958) and Leidl (1958) recorded that there was no difference in the fertility of offspring from inseminated and naturally mated cows.

A possible disadvantage of the use of artificial insemination

is the risk of inbreeding that it carries. In Sweden the number of cows which may be inseminated by a particular bull is restricted by law:- one bull has to be provided for every 1000 cows inseminated and 15 per cent of the bulls must be replaced annually. This is designed to prevent inbreeding and to avoid spreading any undesirable genes which a sire might be transmitting. The studies on gonadal hypoplasia by Eriksson (1943) emphasized the need for such control measures; this condition had become more widespread by breeding from selected high butter fat record animals, which carried this defect. There are no reports on widespread test mating for recessive factors but of recent years increasing attention has been paid to the collection of data on abnormal calves produced (Van Dieten, 1956; Smith, 1958). Although Foote, Henderson and Bratton (1956) maintained that these would be detected in the normal insemination service, attention should also be paid to the detection of the less obvious non-lethal defects in conformation, that could shorten the production life of an animal.

B. Assessment of artificial insemination results

Centre managements have devised methods of measuring the effectiveness of the service as soon as possible after insemination. The accepted practice is to estimate results on a non-return basis, i.e., the percentage of cows and heifers not reported as requiring a repeat insemination within a given period after the first insemination. In Great Britain, until recently, this period was taken as three months from the last day of the month in which the insemination was performed; it was referred to officially as the 3 months' non-return conception rate, and was similar to the 90-120 day figure

reported elsewhere.

The non-return method of assessment is used almost universally although, in the U.S.A., the period has been reduced to 60 to 90 days. More recently, the Milk Marketing Board (Report, 1959a) announced that in the future an even shorter interval, i.e. 30 to 60 days, will be used and this will be referred to as the 30-to-60-day non-return percentage or non-return rate. It is claimed that this will be as accurate as the previous 90-to-120-day figure and that it will have the additional advantage of being available more quickly. However, it must be noted that, in the event of a foot-and-mouth disease outbreak causing a temporary suspension of the service and thereby precluding the farmer from obtaining a repeat insemination, an unusually high non-return percentage will be obtained if this method is used. With a 90-to-120-day conception rate estimation the service would normally be resumed to allow for such a repeat request to be recorded before the final rate had been calculated. The non-return rate is not an exact measure of fertility since the farmer may fail to take advantage of even one free repeat insemination if the first, or paid insemination, was not successful. Although it is difficult to establish on a large number of animals, the difference between this non-return figure and the actual pregnancy rate appears to be approximately 10 per cent (see table 3).

TABLE 3

DIFFERENCE BETWEEN "NON-RETURN" AND ACTUAL CONCEPTION RATES

Period	Reference	No. of first inseminations	Per cent not reported to have required a repeat insemination at 3 months	Per cent actually pregnant	Difference
1947	Clarke (1949)	19,124	62.6	53.4	-9.2
1944-45	Stewart (1950)	2,046	66.1	57.5	-8.6
1946	Stewart (1950)	3,828	65.3	52.2	-13.1
1944-48	Holt (1952,a)	19,687	66.6	58.5	-8.1

Stewart (1950) could not trace 10 per cent of the animals inseminated, indicating the difficulty that would be experienced in attempting to use an actual calving rate. Since the history obtained suggested that some of these could be assumed to be pregnant, it would seem that the actual calving rate was probably higher than reported above. A pregnancy rate of 52.9 per cent from 4,286 first inseminations with a 90-to-120-day non-return rate of 55.7 per cent has been reported from the U.S.A. (Report, 1950). A similar difference between the non-return and actual pregnancy rate was found by Bonadonna (1950, b) The non-return rates in England and Wales (see table 3) were higher than those reported elsewhere but the actual pregnancy rates to a first insemination were similar.

Although the conception rates following second and third inseminations are rarely published, actual pregnancy differences of 9.2 per cent and 5.4 per cent between the first and second inseminations

were quoted by Holt (1952a) and Stewart (1950), respectively. It is generally accepted that 85 per cent of all cows should conceive to 3 or fewer inseminations.

The non-return conception rate is now regarded as a measure of the efficiency of a centre, of an operator, of a bull or of a sub-centre. This assessment has not been shown to be valid for making comparisons between centres.

Recently Jenichen and Zelfel (1959) suggested an alternative method for determining conception rates, the records being assessed on a herd basis. While helping the analysis of herd records, this would be of limited value for assessing the overall efficiency of the insemination centre. Another record, which is of value in assessing the results of a centre, is the number of repeat inseminations relative to the first inseminations. This ratio is normally 1 : 1.4. A check on the number of repeat insemination requests readily gives a measure of the fertility results in the field and of the extent to which farmers are relying upon the service provided.

C. Comparison of the efficiency of artificial insemination and natural service

Since artificial insemination is used over such a wide scatter of herds, comparable data, from an equally large group of herds mated by natural service, are not readily available. In a study limited to one herd, Foot and Ridler (1949) found no difference in fertility over a period of five years of natural mating followed by five years of mating by artificial insemination. Similar results were obtained

in an extensive study of data obtained from herdbooks by Van Oers (1955).

The Milk Marketing Board (Report, 1952) gave data obtained during the years 1946 - 1951 in a group of milk recorded and, therefore, selected herds. The conception rates were presented on the usual 3 month non-return basis (see table 4).

TABLE 4

COMPARISON OF CONCEPTION RATES WITH NATURAL SERVICE AND ARTIFICIAL INSEMINATION

(Report, 1952)

Year	No. of herds	No. of cows and heifers	Natural service conception rate per cent	Overall A.I. conception rate per cent
1946	112	3,420	69.9	-
1947	112	3,970	70.7	-
1948	112	3,676	69.1	-
1949	470	Not given	74.8	64.0
1950	470	" "	73.6	67.4
1951	470	" "	72.6	68.4

It must be remembered that the artificial insemination conception rates were obtained over a large number of herds with different levels of management, whereas, herds, on which natural mating was used, were a selected sample of recorded herds, in which management would, therefore,

be above average.

In a similar study (Stewart, 1952) four groups of records of natural mating had, over a period of 25 years prior to 1949, a 112 day non-return conception rate of 67 per cent with 2,264 first services, whereas with insemination over the period 1944 - 1946 the 112 day non-return conception rate was 65 per cent. There was, therefore, again a tendency for the results with natural mating to be higher than those recorded with artificial insemination. Whether this tendency would be confirmed if strictly comparable data could be used is not known.

D. Limits of fertility

The fact that the average overall 3 month non-return conception rate has remained under 70 per cent, with only a few bulls reaching 75 per cent, has been interpreted by Walton (1958) as indicating that fertility is limited by the potential fertility of the cow. In the data presented by Walton (1958), it was shown that, in artificial insemination, the distribution of the bulls in terms of fertility was not uniformly about the mean. This, it was claimed, was due partly to the fact that low fertility bulls had been eliminated by tests carried out prior to acceptance at the centre and partly to the absence of bulls with conception rates of over 75 per cent. Laing (1949) and Tanabe and Casida (1949) have shown that this limit on fertility is due to early foetal death and not to fertilisation failure. The former appears to occur to a greater extent in bulls of lowered fertility and the bull has been claimed to be one cause

of the delayed return to heat (Salisbury, Bratton and Foote, 1952). Although these workers postulated that the delayed return was an indication of early embryonic mortality, Bearden, Hansel and Bratton (1956), in an extensive investigation, were unable to prove that the embryonic death rate was significantly greater in the low than in the high fertility bulls. Their findings were, however, strongly suggestive of this. An indication of a possible genetic cause of this limit on fertility has been suggested by Ashton (1959), who reported on the possible incompatability of the blood plasma proteins of sire and dam.

E. Planning field trials

In view of the possible influences on non-return conception rates there is an obvious need for care in planning field fertility investigations into insemination techniques. The split-sample technique was said, by Flerchinger and Darroch (1956) and by Kok (1954), to give a definite gain in efficiency. By this method each semen ejaculate for use in the field is split into two or more parts after collection, each part being submitted to one or other of the treatments under investigation. Willett, Ohms and Torrie (1955) brought evidence to show that a collection could be split into several parts for different treatments, provided that at least 80 first inseminations were done with each part. However, this would depend largely on the volume of the ejaculate that could be obtained from each bull per collection day. For example, Hafs, Bratton, Henderson and Foote (1958) have shown that, in studying the effect of a 50 per cent variation in the number of motile spermatozoa per ejaculate, 10 bulls are required per treat-

ment and five ejaculates per bull.

F. Summary

Although the practice of artificial insemination is now widely established the continual expansion of this service has prompted research into improvement of the techniques. Particular attention has been paid to the collection, assessment, dilution and storage of the semen, in order to ensure the widest and most economical use of the sires selected on the basis of progeny tests. The reports of these various investigations have been considered in detail. The effects of herd management, individual bulls, individual techniques, and seasonal effects on conception rates are also discussed.

S E C T I O N II

MANAGEMENT, SELECTION, LENGTH OF USEFULNESS AND HEALTH OF BULLS

A. MANAGEMENT:-

(i) Housing:

There are few publications dealing with the housing of bulls at artificial insemination centres. The important considerations were discussed in the British Veterinary Association Report on Artificial Insemination (Report 1953,d). Most centre managements would agree that, ideally, the bulls should be housed in loose boxes. The tying of the bulls in standings can be satisfactory, provided that sufficient attention is paid to the size of the standing and to the exercising of the bulls. Tying the bulls saves considerably in costs of buildings and also in labour - two very important considerations at a modern centre, which, in addition to having bulls for current use, has also to accommodate bulls awaiting the results of their progeny tests. Further information would appear to be required on the methods of housing bulls that are being held for 2-3 years until their progeny records are available. There is little information on the effect of long periods without services or collections on the temperament, on the semen production or on the fertility of such bulls.

(ii) Exercise:

Whilst it would generally be accepted that exercise is important for general health, bulls at many insemination centres have

remained in good health and have maintained a satisfactory level of semen production and fertility when allowed only the freedom of their boxes. A controlled study of the effect of forced exercise on 8 high- and 3 low- fertility bulls, housed in stalls or boxes, was carried out by Snyder and Ralston (1955). Equal numbers of Guernsey and Friesian bulls served as controls and others were put on a mechanical exerciser for periods increasing from 15 to 30 minutes daily. The quality of the semen from the exercised bulls did not differ significantly from that from the controls. No significant differences in conception rates were recorded with 17,872 inseminations from the exercised group and with 15,420 inseminations from the control group except in the case of the high fertility Guernsey bulls. However, even the difference in these could not definitely be attributed to lack of exercise. In this study, which lasted 6 months, exercise did not result in any improvement in libido, fertility or general health of the bulls. With bulls tied continuously for long periods in standings, a different result might be expected. Although ankylosis of the vertebral joints (Spondylosis) was demonstrated post-mortem in 31 out of 37 bulls examined (Milk Marketing Board, Report, 1957), there was no evidence that the condition was due to the lack of exercise or that it was more common in insemination centre bulls. Reference was made to "Standings Disease" a spastic hind leg condition which was sometimes found associated with spondylosis. Roberts (1953) reported similar findings in a study of 33 bulls. A beneficial effect of exercise on

bulls has been referred to by Bonadonna (1956). Sørensen and Hansen (1950) in a study involving 18 bulls reported that exercise improved semen volume and density. Since the exercise was allowed for only 45 minutes prior to each of the three collections each week, the effect could have been due to a pre-collection stimulus rather than to the actual exercise.

(iii) Feeding:

Considerable attention has been paid to the effect of both the constituents and level of the feed on sexual maturity, on fertility and on semen production. Jones, Dougherty and Haag (1945) demonstrated a delay in sexual maturity in young bulls raised on alfalfa hay, but in view of subsequent work it is doubtful if this could be attributed to reduced energy intake. Bane (1954), using 6 pairs of identical twin bulls reared on different planes of nutrition from 1 to 18 months of age and subsequently put on uniform diets, could not demonstrate any lifetime effect on semen characteristics or on libido. No data were given on the examination of the semen prior to placing these animals on a uniform diet. James (1950), using 5 pairs of identical twin bulls fed on two different nutritional levels from 20 weeks to 2 years of age, found that at 15 to 24 months of age the bulls in the low-plane group had a reduced level of total spermatozoal production and had smaller testes than those in the high-plane group. No data were given on the subsequent performance of these bulls. Davies, Mann and Rowson (1957), using one set of identical twin calves, reported reduced spermatozoal production and a retarded onset of spermatogenesis

in the calf on the low-plane of nutrition. No fertility measurement was included in the above studies but Bratton, Musgrave, Dunn, Foote and Henderson (1956b) found no significant differences in conception rates among bulls reared on high, normal and subnormal nutritional levels. However, in the last group, the number of motile spermatozoa per ejaculate was reduced. Flipse, Snyder, Thacker and Almquist (1953) reported that the age at which the first viable spermatozoa were obtained was delayed in bulls reared on only 70 per cent of the normal feed level, whilst Baker, Van Demark and Salisbury (1955a) reported that numbers of spermatozoa per ejaculate were significantly related to the age and weight of the bull. Flipse, Almquist and Johnson (1956) found that, when bulls were reared on a diet containing dried skim milk, the ejaculate volume was adversely affected; if liquid skim was used instead the ejaculate volume was normal and, furthermore, the number of spermatozoa per ml. was increased. Although the above evidence suggests that the level of feeding adopted in rearing a bull may affect its capacity for semen production, there is no clear proof of a corresponding influence on fertility. A relationship between fertility and the measurement of certain characteristics of semen could not be assumed unless the differences were very marked, and in most of the above reports they were not.

In adult bulls several studies have been reported on the effect of feed level, and of type of ration on semen production, libido and fertility. Mann and Walton (1953), using one bull, recorded no effect on the usual semen characteristics following restriction of food intake

to such an extent as to reduce body weight; there was, however, a marked reduction in the accessory gland secretions. There have been many reports on the use of various food constituents in the ration. The British Veterinary Association (Report 1953,d) recommended that the nitrogenous: carbohydrate ratio of the concentrate mixture should lie between 1:3 and 1:4. The effect on conception rate of including animal protein in the form of skim milk powder in the concentrate mix was investigated by Branton, Bratton and Salisbury (1949) who, on the basis of the resultant fertility of the bulls, did not find this superior to vegetable protein (corn gluten feed or soyabean oil meal). Similar results were reported by Prabhu, Guha and Bhattacharya (1953) who used blood meal as animal protein. Schmidt (1953) found an increase in spermatozoal numbers and lowered ejaculate volume when a supplement of 8 litres skim milk a day was given to each bull, but he considered that the reduction in ejaculate volume was due to feeding skim milk.

Most centres are opposed to the feeding of large quantities of silage because it is considered, although no controlled experiments have been carried out, that it has a detrimental effect on libido and causes distension of the abdomen. The feeding of large amounts of grass silage for 1 to 2 years has, however, been found to have no detrimental effect on fertility or on semen quality (Quicke, Phillips and Dreher, 1950; Flipse and Almquist, 1957). Flipse and Almquist (1957) concluded that, under the conditions pertaining, a high grass silage ration of 4 lbs. per 100 lbs. body weight could result in a

29 per cent saving in feed costs compared with the conventional hay-concentrate ration. It is quite possible, however, that other types of silage may have a different effect, but Flipse (1957) reported negative results with corn silage in the rearing of identical twin bulls.

The view is widely held that young spring grass has an adverse effect on semen quality but no controlled studies of this have been reported. Kordts (1955), in an investigation into the effect of various green foodstuffs in the ration, found that a sudden change from stall feeding to purely grass fodder led to a reduction in spermatozoal survival but, apart from this, there was no adverse affect on semen volume or quality. No fertility study was reported. Using 5 pairs of identical twin bulls, De Groot (1958), after feeding one kilo. of dried grass daily in place of part of the concentrate ration fed to the control group, observed no effect on semen characteristics. Although in the experimental group an increase of libido was noticed, this was only in the first month of use of the dried grass. Flipse and Almquist (1954) reported previously no effect on overall fertility (based on 21,231 first inseminations) or on semen quality when 2 lb. of the usual hay ration was replaced by an equal quantity of dried grass. However, they observed an improved fertility in 2 of the 18 bulls on the dried grass supplement, indicating that it may have some beneficial effect on bulls of low fertility, on bulls kept on poor quality diets, or on bulls being heavily used. Branton, Patrick, Newsom and D'Arsenbourg (1953b) found that with grazing bulls at pasture,

although this was cheaper than dry feeding, there was no significant improvement in conception rate or semen quality. In general, therefore, in view of these results the expense of feeding fresh or dried grass to bulls does not appear to be justified.

Of the vitamins, vitamins A and D appear to be the most important in connection with the fertility of bulls. Under normal conditions at centres a vitamin deficiency should not occur. The adverse effects of Vitamin A deficiency on service behaviour and spermatozoal motility were observed by Bratton, Salisbury, Tanabe, Branton, Mercier and Loosli (1948) in 6 mature dairy bulls, but only after they had been fed a carotene and Vitamin A-deficient diet for 2 months. However, when good hay is fed this is unlikely to occur.

(iv) Handling of bulls:

Since there are great variations in their behaviour and temperament, a rigid system cannot be laid down and be expected to be equally satisfactory for all bulls. The chief aim should be to accustom the bull to a set routine for semen collections, bearing in mind the different characteristics of the individual. Little need be said about the handling of the bulls for cleaning and feeding. At collection times, however, the main aim is to stimulate the bull to mount the teaser and to ejaculate, by handling it in the manner which has been found to initiate this conditioned reflex. (See page 28).

B. SELECTION OF BULLS:-

This will be discussed only briefly since a review of the methods of progeny testing and sire appraisal is outside the scope of this communication.

Whilst the genetic improvement in the quality of livestock following the early use of artificial insemination was disappointing, the improved methods of selection of bulls should ensure that the primary object of the insemination service, i.e. livestock improvement, will be fulfilled in the future. The disappointing lactation records of over 1,400 heifers, got by the early use of artificial insemination in England, as analysed by Robertson and Rendel (1954), indicated a need for a re-appraisal of the methods of bull selection; the yields of daughters got by artificial insemination and natural mating within the same herds were not significantly different and this applied to herds at all levels of production. Significant differences were found between progeny groups and this emphasised the need for attention to be paid to both progeny testing and alternative methods of bull selection. Similar findings have been reported by workers in other countries (Wadell and McGilliard, 1959). However, the improvement in production in New Zealand (Report, 1956,c), especially when the insemination bulls were very carefully selected, did confirm the possible advantages of using artificial insemination. Robertson and Rendel (1954), in the light of their experience with the early progeny records, outlined the contemporary comparison method of assessing progeny tests carried out under routine conditions. This method was reported on in detail by McArthur (1954) and has formed the basis of the methods of bull appraisal and selection outlined by the Milk Marketing Board (Report (1953a).

The use of progeny testing stations, as adopted in, for example,

Denmark, was studied and comparisons of results obtained by both methods in England and Wales were made by the Milk Marketing Board, (Report, 1954 a) The present plan is to select, for cattle breeding centres, sons of bulls which have been progeny tested or, if age and fitness of the bull permit, to use the proven bull itself. The above measures only refer to production, but inspection of a bull's daughters, half sisters and dam for type is an important factor in bull selection. The view expressed by Foote, Henderson and Bratton, (1956) that selection for production alone will not appreciably affect type is not generally accepted (Freeman and Dunbar, 1955). The genetic relationship between type and production is, however, being also investigated (Milk Marketing Board, Report, 1954 a)

C. TESTING FOR RECESSIVE GENES:-

The detecting of carriers of recessive lethal or otherwise harmful genes has assumed increasing importance in view of the more widespread use of progeny tested sires. Rollinson (1955) reviewed the information, then available, on hereditary factors influencing reproductive efficiency and he concluded that the loss was not great from easily defined conditions such as White Heifer disease. In the case of such defects as nymphomania and impotentia coeundi, however, the loss, attributable to heredity, was not known. Selection for fertility would appear to be of doubtful value in the present state of our knowledge. The position regarding abnormalities, attributable to lethal genes, has been reviewed by Gilmore (1949), by Gotze (1952) and by Gotink, De Groot and Stegenga (1955). Examples of defects of

economic importance are oedema of calves in the Ayrshire breed and dwarfism in the Hereford breed. Robertson (1954) suggested that an artificial insemination bull could be bred to 20 of its own daughters in order to ascertain if it was a carrier of a recessive factor. Foote et al. (1956) claimed that information on the transmission of lethal genes could be accurately obtained by checking the calves of the 1,000 cows used in its progeny test; however, with this method it was admitted that the results would be more difficult to obtain. The incidence of gonadal hypoplasia in the breeding herds of the Swedish Highland breed decreased over a period of nine years from 15.2 per cent to 9.4 per cent as a result of control of use of infected bulls and culling of infected cows after clinical examination (Eriksson, 1943; Lagerlof and Settergren, 1952). However, the report of Lagerlof and Boyd (1952) indicated that, especially in certain areas, this condition was widespread in the commercial herds in which there had been little culling of infected females, although the bulls used had been, in the main, clear of this factor. This report indicated that in this condition complete control could not be effected through the bulls. Also no mention was made of test mating bulls for this factor.

D. HEALTH TESTS AND DISEASE CONTROL:-

Since the secondary aim of insemination is disease control, there is an obvious need, and also a definite onus on the insemination centres, to ensure that bulls are free from infectious disease prior to their introduction into the centre and that they remain disease-free while they are at the centre (Rasbech, 1956; Bartlett, 1956).

Particular attention has been paid to the elimination of tuberculosis, brucellosis, trichomoniasis and vibriosis, while, in certain countries, the control of leptospirosis is also receiving attention. Although there is no extensive information on the incidence of Johne's disease in insemination bulls, there would appear to be a definite risk of this being introduced into a centre. Smith (1958) reported that 3 out of 102 disposals were on account of Johne's disease, thus emphasising the need for precaution against the spread of this infection into and within the centre. The practice of exchanging bulls between centres is sometimes adopted but is not desirable where there are disease risks. Moreover, with the increasing emphasis on progeny testing, other centres may be reluctant to use such bulls in preference to progeny testing other young bulls for themselves.

E. BLOOD TYPING:-

The available information on blood grouping was reviewed by Neimann-Sørensen (1958). Parentage checks, based on blood group identification, are widely made in the U.S.A., Canada, Scandinavia and also in England and Wales in connection with progeny tested bulls and special matings (see Milk Marketing Board; Report, 1956,a). It is also possible that, as extensive data on this are accumulated, certain blood groups may be found to be related to such factors as production characteristics. The investigations of Ashton (1958) into blood protein composition in the different breeds may also yield similar information.

F. PERIOD OF USEFULNESS OF BULLS AT ARTIFICIAL INSEMINATION CENTRES AND DISPOSAL RATES:-

Available data indicates that the length of life of a bull

at a centre is under 3 years; this, however, is dependent on the age at which bulls are brought to the centre and also on the extent to which culling is practised. The Milk Marketing Board (Report, 1957) showed that the 542 bulls purchased during the period 1944 to 1950 had an average length of service of 4 years and 9 months; 237 of these bulls were under 2 years of age and a further 61 between 2 and 3 years at time of purchase. An indication of the reasons for disposal was given by Smith (1958) who reported that 102 bulls were discarded from the centres during the year ended 31st March 1958 for the following reasons:-

progeny below standard, 43;

poor conformation of bull, 2;

reproductive failure (including poor semen production, poor service behaviour, and low conception rate), 30;

accident or disease, 19;

miscellaneous reasons, 8;

The chief cause of the disposals was, therefore, unsatisfactory progeny reports. Disposals in the U.S.A. were classified by Bartlett (1956) as follows:-

died, 15 per cent;

sold for slaughter because of low efficiency, 45.6 per cent;

various reasons, 39.4 per cent;

No details of these classifications were given but, here again, poor progeny results appeared to account for a large proportion of the

disposals. It is also noteworthy that disease accounts for the minority of the disposals, indicating the efficiency of both the pre-entry tests and within-centre health control.

SECTION III

SEMEN COLLECTION TECHNIQUES, COLLECTION FREQUENCY, AND FACTORS AFFECTING SEMEN PRODUCTION AND LIBIDO

A. Semen collection technique

(i) Artificial vagina method. This is the universal method for the routine collection of semen and the equipment used is, in the main, similar to that described several years ago by Anderson (1945), Hammond et al. (1947) and Herman and Madden (1950). Most centres prefer to use the short pattern artificial vagina (Walton, 1945) to ensure that the semen is ejaculated directly into the cone or collecting tube and not on to the liner. Any preputial debris that may be carried on to the liner at the time of ejaculation is, therefore, not mixed with the semen. An insulating muff is placed around the collecting tube to protect the semen from cold shock and this also prevents breakage of the tube. In cold weather, insulation of the cone may be necessary. In recent years, an increasing amount of attention has been given to the sterilisation of the artificial vagina in order to prevent any possible spread of infection between bulls within a centre. The safest practice, used in most centres, is to keep separate equipment for each bull. The use of a roughened type of liner has now been more widely adopted; although there is little published data on this, the generally accepted view is that such a liner ensures a more constant service behaviour in a bull. The reaction of the penis to

the application of the artificial vagina has been described by Bonadonna (1956), who stressed the importance of applying the artificial vagina at the correct time. However, as there is considerable bull to bull variation in the state of the penis at the time of ejaculation, it is difficult to define the correct time for this.

In spite of the report of Bane (1950) that latex rubber may have a spermicidal action, equipment made of this has continued to be widely used although care is taken over cleansing same. Kordts (1956) described the new "Kiel" model of artificial vagina which was made of plastic material. It had an inner plastic tube, which could be discarded after each collection thus obviating the need for cleaning and sterilising of cones. A means of altering the internal pressure during service was reported by Sobek (1955), who claimed that this gave increased ejaculate volumes. A more elaborate modification of the standard type of artificial vagina was described by Millar (1958); this was designed to ensure adequate stimulation of the ejaculation reflex and to prevent temperature shock in the raw semen. It has not been extensively used and, although it appears to incorporate many desirable features, it may prove to be too complicated for routine use in view of the time required for taking it apart, cleansing and re-assembling it. While vaseline is commonly used as a lubricant, Wohlfarth (1957) attributed an outbreak of granular and vesicular preputial lesions to the use of yellow vaseline, the condition apparently disappearing when milk was used as a lubricant.

Although Rowson (1947) claimed that semen could be obtained by

means of a urethral fistula, such a technique has not apparently been used elsewhere.

(ii) Electro-ejaculation. The technique, devised originally by Gunn (1936), for the electrical stimulation of ejaculation in the ram was successfully modified for use in the bull by Laplaud and Cassou (1948), who used a bi-polar rectal electrode, and by Thibault, Laplaud and Ortavant (1948), who used a single electrode with a series of 30 rings thereon connected to the potentiometer. The apparatus, which used a current of 50 cycles per second, was further modified by Rowson and Murdoch (1954), who reported on the use of two simple finger electrodes. These were introduced into the rectum on two fingers, the operator's hand being covered by a rubber glove. By means of the finger electrode it was claimed that the stimulus could be applied more directly to the ampullae and seminal vesicles. Manton (1956) reported a 3 month non-return conception rate of 68% to 2,923 first inseminations with electro-ejaculated semen from one Hereford bull, which compared favourably with 65% with semen collected by the standard artificial vagina from the same bull and used for 1,218 first inseminations. Similar results were obtained with one Dairy Shorthorn bull over 1,000 first inseminations. These authors claimed that the semen obtained by electro-ejaculation was generally of good quality. The recommended method of application of the electrical stimulus was by increasing the voltage pulses over a period of 3 to 5 minutes. An alternative apparatus, using a rectal probe with four longitudinal brass electrodes set into it, was described by Marden (1954). The optimum

frequency of the current was given as 50 cycles per second but Dziuk, Graham and Petersen (1954), who used it extensively, found no difference in the efficacy of frequencies from 15 to 90 cycles. They finally used 60 cycles in their investigation, which revealed no difference in conception rates with electrically ejaculated and naturally ejaculated semen. Erection and protrusion of the penis appears to occur in a high percentage of the bulls used and the semen quality, although lower in spermatozoal density, is satisfactory. The general reaction to the treatment appears to be variable and occasionally the bull may go off its feet. The technique has been investigated by many other workers including Knight (1955), Laurans (1952), Van Rensburg and De Vos (1957) and Rollinson (1956). More recently Parraud, Aquerreta, Tewes and Galan (1958) reported on the successful use of a finger electrode apparatus with an increased frequency (115 cycles per second) on 1900 bulls under field conditions.

An electro-ejaculator is undoubtedly of use in older bulls, in which a lack of libido or inability to mount has developed, but its use in younger bulls should be carefully considered in case the failure to mount and ejaculate normally is due to genetic causes. The possible risk of injury to the bull due to a general reaction with such treatment must also be borne in mind.

(iii) Pre-collection stimulation. The evidence indicating that pre-collection stimulation has considerable effect on semen quality is largely circumstantial. Hellstrom (1947) obtained increased ejaculate volume by restraining the bulls for a few minutes before allowing them

to serve into the artificial vagina. Similar findings were reported by Collins, Bratton and Henderson (1951) and by Branton, D'Arsenbourg and Johnston (1952), who obtained an increase in the total number, and also in the numbers of motile spermatozoa, with this treatment. Crombach, De Rover and De Groot (1956), in a more critical trial with two pairs of identical twin bulls over two months, found a 100 per cent increase in the numbers of motile spermatozoa when the bulls were restrained for 5 minutes and then caused to mount falsely before the first ejaculation was taken. No details of the semen examinations or fertility studies were reported. An explanation for this was put forward by Parsutin (1956), who claimed that excitement brought about a more complete contraction of the epididymis. The possible effect of the pre-collection stimulation on conception rates was described by Kerruish (1955). With 10 bulls used over a period of 5 months without pre-collection treatment a conception rate of 60.8 per cent was obtained. Pre-collection stimulation was carried out over the next 5 months on these bulls. Bulky food and water were withheld from the bulls for 12 hours prior to collection and the teaser was also changed. This treatment resulted in a conception rate of 68.5 per cent. The experiment was not strictly controlled since the two sets of fertility results were obtained at different periods.

Despite the absence of adequate fertility data on the effect of pre-collection stimulation, there appears to be sufficient evidence of a beneficial effect on semen production to justify it.

(iv) Collection frequency. As the maximum collection frequency compatible

with a satisfactory conception rate is of economic importance in commercial artificial insemination, several workers have considered it advisable to repeat, on a larger scale, the investigations of earlier workers. Anderson (1945) classified these investigations into two groups; those in which there was an interval of minutes between ejaculation, and those in which there was an interval of days. Although most workers confirmed the early findings of Kirillov and Morozov (1937) that increased collection frequency appeared to result in increased spermatogenesis, no definite ejaculation frequencies were recommended, and some of the original studies were confined to a few bulls only. It was clear that before frequency of collection studies could be accurately assessed, the bull's capacity for semen production and the effect of stimulation prior to collection would have to be considered. Moreover, there was a need for fertility studies with semen obtained at different collection frequencies. Patrick, Branton and Newsom (1949), studying an ejaculation pattern of one every 4 days, two every 8 days or three every 12 days, found no difference either in the percentage of suitable ejaculates or in the 30 to 60 day non-return percentage. On the other hand Mercier, Bratton and Salisbury (1949), using 12 bulls, obtained 45 per cent more usable semen with bulls ejaculated every 6 days, compared with those ejaculated twice every 12 or thrice every 18 days; certain fertility differences were ignored as the authors considered they were due to the grouping of the bulls and not to the treatment.

These previous studies were carried out mostly with older bulls

but Baker, Van Demark and Salisbury (1955,b) used 9 young bulls for one year after puberty. No restraint was effected before allowing the bull to mount and ejaculate. The effects on libido, as well as on certain semen characteristics, were measured in bulls on ejaculation frequencies of 1, 2 or 3 times per week. No effects were noted on semen volume, spermatozoal concentration, percentage of motile or abnormal spermatozoa, or on the total spermatozoa per ejaculate. Libido was reduced in 2 of the 3 bulls in the 3 ejaculations per week group. One bull was maintained on this schedule for a total of 4 years, which confirmed that this bull had a great potential for spermatozoal production.

The quantitative aspects of spermatogenesis were reviewed by Van Demark (1956), who showed how an increased frequency of collection, combined with careful attention to pre-collection stimulation, could make an increased number of spermatozoa available. Further partial semen exhaustion tests at intervals of one, four and seven days suggested there was a rapid replenishment of spermatozoal reserves during the first 24 hours after the collection, and a steady rate thereafter up to 7 days. In an investigation commenced in 1948, Bratton and Foote (1954) showed that with one ejaculate every four days or one every eight days, over a 272 day period, the 60 to 90 day non-return conception rates, based on 55,350 first inseminations, were 61.8 per cent and 64.8 percent respectively; the conception rate difference was not significant at the 5 per cent level. The average numbers of motile spermatozoa, produced every eight days, were 11.4×10^9 and 7.0×10^9 respectively,

there being a 63 per cent increase with the more frequent collections. Subsequently with the same bulls, when one ejaculate every four days and two every eight days were compared, no effect on conception rates was obtained, and two ejaculations every eight days yielded approximately 60 per cent more motile spermatozoa than did one ejaculation every eight days with the same bulls.

In a second investigation by the same workers (Bratton, Foote, and Henderson 1954, b), the effect of restraining the bull for 20 minutes prior to taking the first ejaculation, followed by 10 minutes rest and 10 minutes restraint before the second ejaculation, gave similar results, the conception rates being 73.3 per cent and 73.4 per cent for ejaculations at one in 8 and two in 8 days respectively. This investigation was made in 1952 and no explanation was given for the increase of 10 per cent and more in conception rate over the 1948 figures. As no anti-biotics were used in the diluents in the early trial the suppression of some infection could have been responsible for the difference in results.

In a survey of possible methods of measuring the sexual behaviour and semen production potential, Almquist and Hale (1956), in order to confirm the above reports, made certain observations under standard conditions as a preliminary to their main studies on ejaculation frequency; these were aimed at assessing under standard conditions the reaction times for the bull, and the number of ejaculations required to deplete the semen store. The reaction time, as measured at the collection of the first ejaculate, was shown to be an unreliable

method of determining sexual activity. An accurate measurement was obtained by observing the number of ejaculations that could be collected within 15-20 minutes; the interval between recovery of the urge to ejaculate and the effect of a new stimulus (or teaser animal) were also observed. The need for using standard conditions of stimulus was thereby demonstrated. No effect was recorded when oestrous mucus or wormwood oil was used in an attempt to stimulate the bull, a finding in contrast with the report of Hart, Mead and Regan (1946), that the odour from the oestrous secretion may have a stimulatory effect. Whilst these procedures could not be adopted as a routine at an artificial insemination centre, they were an attempt to obtain basic information which might be of practical value. In an attempt to measure the available reserves of spermatozoa, 44 depletion trials were carried out by Almquist and Hale (1956) with 21 bulls. The bulls were allowed to ejaculate without restraint or encouragement between ejaculations except that in some trials a fresh teaser was used to shorten the reaction time. The results, based on the total number of spermatozoa obtained in the first 20 ejaculates, showed that, while 31 per cent of the total spermatozoa collected were obtained in the first 2 ejaculates, 76 per cent were collected in the first 10 ejaculates. The average total number of spermatozoa obtained over the 44 trials with bulls of different ages was 36.6×10^9. In the older bulls, this number exceeded 50×10^9 spermatozoa. Whilst the total spermatozoa obtained in a trial can only be taken as a minimal estimate of the reserves, Almquist, Amann

and O'Dell (1958) subsequently confirmed, by post-mortem counts of spermatozoa in the genital tract, that this depletion trial gave a reliable estimate. However, there are indications that the numbers of spermatozoa ejaculated fall to, but not below, a certain low threshold level after several successive ejaculations. The replacement of the spermatozoal reserves was found to be completed within one week if 11 to 30 successive ejaculates were collected, whereas, when the number of ejaculates exceeded 30, the spermatozoal concentration was still below the pre-depletion level at the end of this period.

The important question as to the effect of ejaculation frequency on semen output and sexual behaviour was the next to be dealt with by Almquist and Hale (1956). They studied 15 mature bulls on a once per week collection schedule for 12 months and a twice per week rota for the next 12 months, the bulls being restrained for 4 - 5 minutes before ejaculation. Doubling the number of collections increased the weekly output of motile spermatozoa by 67 per cent (from 7.9×10^9 on the once weekly rota to 13.2×10^9 on the twice weekly rota) with no appreciable change in the 60 to 90-day non-return rate. This experiment was not strictly controlled as the two treatments were performed on the same bulls in successive years and the bulls were all one year older when put on to the twice per week rota. However, statistical analysis did not suggest any significant effect of the age of the bull on these results. The number of ejaculations per collection day was not constant, the average range being 1.0 to 1.3

on the once weekly rota and 2.2 to 3.0 for the twice weekly rota; presumably this was due to the semen requirements for field insemination, more ejaculates apparently being required with the twice weekly collection rota.

Using two groups each of 5 bulls, incomplete data on the comparison of two ejaculations on one day per week with two ejaculations on three days per week indicated that double the number of motile spermatozoa was obtained per week with the second treatment. Each bull was kept on each treatment for 24 weeks. In the case of one bull, which was ejaculated 6 times per week, a 47 per cent reduction in spermatozoal count, and a 13 per cent reduction in ejaculate volume was recorded, indicating that certain bulls could not be ejaculated so frequently for extended periods. A similar effect was found in sexual behaviour. When giving two ejaculations per week only 3 of the 10 bulls required a new stimulus, whereas, on the 6 ejaculations per week, 7 of the 10 bulls required a new stimulus over the 24 week period. Although it was claimed that the weekly spermatozoal output was increased by 112 per cent by making six, rather than two, collections per week, more frequent changes in the teasing routine were required to maintain the bulls' sexual activity. There was no information on the effect on fertility of the use of such a procedure over 24 weeks, since apparently no inseminations were performed with the semen. However, these studies gave some indication of the potential semen production of a bull over a limited period, which could be of practical assistance as in progeny testing a young bull

in the minimum time. ~~However,~~ Haq (1949) suggested that the use of an immature bull could set up testicular degeneration, but this observation, which was based on the finding that 60 per cent of 41 bulls tested for infertility had been put to service at 9 to 12 months of age, would require to be confirmed over a larger bull population.

Boyd and Van Demark (1957) confirmed the findings that depleted spermatozoal reserves were replaced after 7 days' rest. Similar findings with identical twin bulls were recorded by Frederick (1959). Also, this author, using bulls in routine insemination work, found that 4 ejaculations per week, compared with 2 per week, reduced the sexual interest and semen volume. However, there was an 88 per cent increase in spermatozoal production per week without any adverse effect on fertility. Hafs, Hoyt and Bratton (1959) with daily ejaculation of aged bulls, over a period of 8 months, found no adverse effect on fertility or semen quality.

Although individual bulls vary in their reactions to frequent semen collections, the available evidence indicates that with adequate stimulation the average mature bull could be expected to ejaculate at least 4 times per week. However, extensive data have not been obtained on either the period over which such a collection rota could be maintained, or on the effect on fertility. Most of the work has been carried out on bulls over 20 to 24 months of age.

B. Other factors affecting semen production and sexual behaviour

In the bull the degree of sexual interest and ability to serve

cannot be taken as an index of fertility (Lagerlof, 1934; Anderson, 1939). For example, it is well known that certain slow working, but highly fertile, bulls repeatedly produce semen of good quality. It is essential for artificial insemination operations to maintain libido since there is good circumstantial evidence that, in the main, a higher percentage of usable ejaculates will be obtained. Several of the factors affecting semen production and fertility have already been mentioned. The remainder will now be considered.

(i) Age and size of bull. Bratton, Musgrave, Dunn, Foote and Henderson (1956,b), in their study of growth of bulls up to 80 weeks of age, found that the capacity for semen production increased with age and ~~it~~ was related to body weight increases. Using bulls under 12 months of age, Baker, Van Demark and Salisbury (1955,a) reported similar results. Van Demark (1956) reported on the semen production of a group of 15 Holstein bulls over the first year after puberty and the changes with age and body weight are summarised in table 5.

TABLE 5

CHANGE IN SEMEN PRODUCING CAPACITY OF HOLSTEIN BULLS AFTER PUBERTY (MEAN OF 15 BULLS)

(FROM VAN DEMARK, 1956)

Characteristic	Quarter year period			
	1	2	3	4
Av. body weight (lbs.)	807	984	1,136	1,278
Semen vol. per ejaculate (ml.)	2.34	3.21	3.51	3.36
Sperm. concentration x 10^6 per ml.	429	735	916	987
Total sperm. per ejaculate x 10^6.	1,255	2,690	3,592	3,668

This study clearly demonstrated that there was an increase in semen production with increasing age and increasing body weight; it was unfortunate that no information on any changes in libido were recorded. Van Demark, Boyd and Baker (1956) also presented data, on semen collection from one bull over a 4 year period, indicating that the semen production increase continued beyond the first year after puberty. A significant correlation was also found between the combined weights of the testes and epididymides of each of 11 bulls at slaughter and the total number of spermatozoa produced in 10 consecutive collections taken from them a few days previously, (Van Demark, 1956). Sixty four per cent of the variations in the total spermatozoa obtained were associated with testes and epididymides weight. Van Demark (1956) also presented evidence to show that 80 per cent of the testicular weight variation was associated with body weight, and that a correlation between total spermatozoa produced and body weight in the bulls studied was, therefore, to be expected.

On the basis of 4 exhaustion tests carried out at weekly intervals, Willett and Ohms (1957b) calculated a coefficient of correlation between scrotal circumference and average spermatozoal production of 0.92 ($P<0.01$) for 16 bulls of 13 to 18 months of age and of -0.53 ($P>0.05$) for 6 bulls of 9-10 years of age. Bialy and Smith (1958b), using mature bulls, could not find a significant correlation between the combined weights of testes and epididymides and numbers of spermatozoa recovered from the epididymides at post-mortem examinations.

It would, therefore, appear that, in the first few years of life, there is some correlation between total spermatozoal production as measured by exhaustion tests and either testicular size or body weight. There is a need to determine if such a correlation is shown by older bulls.

Little evidence is available on the effect of age on libido, provided that the bull is sound and that no physical defect prevents it from mounting. For example, in young beef bulls in fat condition for shows the lack of libido, although usually only temporary, can be of importance (Couttie and Hunter, 1956) since in the beef breeds sexual desire tends to be on a lower level than in the dairy breeds.

(ii) Effect of management, transport, and certain chemical and medicinal agents. Although Gould and Bennet (1950) claimed that resting the bulls every 4th month improved semen quality and conception rate, this has not been confirmed from other centres. The improved fertility after the adoption of this procedure may have been the result of other changes, in particular additional younger bulls were brought on to the centre to permit this resting of the bulls.

Meschaks (1953) claimed that increased excretion of neutral steroids in the urine, followed by morphological abnormalities in the spermatozoa and decreased libido, could be produced by the transport of the bulls between centres. Willett (1957) studied the breeding records of 60 mature bulls that had been moved 65 times in all. Although there were some conception rate differences after the moves, they were neither permanent nor significant. Hafez and Bonadonna (1958) found that the average semen volume of 22 Friesian bulls,

imported from 4 different countries, varied with the country of origin. These workers did not appear to appreciate that the characteristics of the individual bulls may have been responsible for this.

The adverse effect of giving chlorinated naphthalene in corn oil on spermatogenesis has been reported by Vlahos, McEntee, Olafson and Hansel (1955) who also demonstrated that such a bull was fertile upon recovery from this. Infra-red radiation was reported by Koriath (1958) to have a doubtful effect on behaviour and semen production. Reference was also made (Report 1960, b) to the possible detrimental effect of sodium iodide therapy on spermatogenesis.

(iii) Seasonal and climatic effects. Early reports were reviewed by Anderson (1945) and by Mercier (1946) who claimed that the seasonal effect on spermatogenesis was largely conditioned by the hours of daylight, temperature variation being of less importance. In a study of 328,295 first insemination results in Canada over a 4 year period, Burgess (1953) found no significant monthly or seasonal differences in conception rates. Schindler (1954,a) found that in Israel conception rate was lowest in September, and in this month the lowest spermatozoal concentration and survival rates were also recorded. Schmidt (1954) reported that hours of daylight and temperature could be correlated with semen quality changes and also with fertility. However, when the seasonal differences in temperature and length of daylight are small, a marked change in semen quality or fertility would not be expected, Marked seasonal differences in the semen

quality over a period of one year have been reported by Johnston and Branton (1953) but there were no corresponding significant differences in non-return conception rates. Casady, Myers and Legates (1953) also obtained an adverse effect on semen quality by subjecting animals to high environmental temperatures (70-99°F). Patrick, Kellgren, Johnston, Hindery, Shelwick and Banksten (1959) subjected animals in the southern part of the U.S.A. to varying conditions of atmospheric temperature and humidity, but no change in semen production or fertility was recorded. In a study of the seasonal effects on fertility over a 5 year period, Erb and Waldo (1952) found that the conception rates (non-return) were highest in September to November and lowest in January to April.

(iv) Genetic effects. Eriksson (1950) suggested that the lack of sexual desire in Swedish bulls was an inherited and, therefore, an undesirable characteristic; this has not been shown to be a hereditary condition in British breeds. Lack of libido, encountered in Aberdeen Angus bulls, has been described by Couttie and Hunter (1956), but methods of rearing may be partly responsible for this. Testicular hypoplasia is known to occur in Great Britain; it has been reported on by Laing and Young (1956) who, although unable to demonstrate conclusively that its origin was genetic, presented evidence that was highly suggestive of this. In these cases spermatogenesis was also often impaired even when a normal sized testicle was present, but often there was no lack of libido. Hypoplasia of the genital organs was extensively studied by Eriksson (1943), who showed it to be hereditary and to affect both males and females of the Swedish Highland Cattle.

S E C T I O N IV

ASSESSMENT OF SEMEN QUALITY

The development of artificial insemination, and in particular the use of single ejaculates from one bull for large numbers of cows, has prompted many workers to continue to search for an accurate and objective method of assessing the potential fertility of a given sample of semen. The economic importance of both the selection of bulls of high fertility and the use of ejaculates that can be relied on to give a high conception rate is appreciated by the insemination organisations. There is little doubt that estimation of the initial motility, of the concentration of the spermatozoa, of the volume of the ejaculate, and of the resistance and viability of the spermatozoa (see review by Anderson, 1945) serve to differentiate the infertile or low fertility bull from the high fertility bull. However, artificial insemination centres require means of detecting slight differences in fertility between bulls and even between different semen samples of the same bull.

Methods of assessment may be grouped under four categories:

A. Microscopical methods, comprising motility and spermatozoal density estimation, differentiation of live/dead spermatozoa, and counting of abnormal forms.

B. Biochemical methods, by which different metabolic changes in the semen are measured.

C. Methods of detecting the susceptibility of the spermatozoa to changes of temperature or to dilution.

D. Physical and other methods, in which the rate of spermatozoal

movement, the associated electrical changes in the semen and other changes are measured objectively.

A. Microscopical Methods

(i) Spermatozoal density. Estimation of spermatozoal density was originally made by using a haemocytometer slide (Walton, 1927). Smith and Mayer (1955) have reported that the use of eosin in the diluting fluid can help to shorten the process. However, where large numbers of samples require to be counted quickly, this method is not always suitable. For routine dilution of semen to give a constant number of spermatozoa per insemination, many workers now prefer to measure the opacity of a spermatozoal suspension either by standard opacity tubes (Salisbury, Beck, Elliott and Willett, 1943, b; Kyaw, 1944), or by an absorptiometer, the opacity of the suspension being proportional to the spermatozoal concentration. The absorptiometer method, which was first used for ram semen density estimation by Comstock and Green (1939), was applied to bull semen by Henle and Zittle (1942), by Salisbury et al. (1943,b) and by Rothschild (1950,b). The accuracy of this method was questioned by Willett (1950), but subsequently Willett and Buckner (1951) reported that by the use of a semen dilution rate of 1:40 sufficiently accurate results could be obtained with a standard absorptiometer calibrated by using spermatozoal suspensions of known density. The use of a standard "Eel" colorimeter was described by Cox and Melrose (1953), who also indicated the desirability of performing occasional check calibrations and advised the regular use of a standard opacity tube to detect any errors due to light variations.

The "Hilger Biochem" absorptiometer was used by Bishop, Campbell, Hancock and Walton (1954), who emphasised the need for allowing the spermatozoal suspension to stand for a few minutes before measuring its opacity in order to allow any flow movement in the suspension to stop. This technique has also been described by Tacken (1952), De Waal, De Bois and Hendrikse (1952) and Ullner and Richardt (1955), who used a compensation photometer. More recently, Hickman (1958) estimated spermatozoal density by measuring the packed cell volume of semen samples which were centrifuged at 10,000 r.p.m. for 10 minutes in haematocrit tubes. This method was checked by Foote (1958), who concluded, after tests with 33 samples from 32 bulls, that, while this method might be sufficiently accurate for clinical work, it could not be considered to be so for use in routine artificial insemination.

Although considerable attention has been paid to methods of estimating the spermatozoal density of semen samples, Cupps, Laben and Mead (1953) and Bishop et al. (1954) failed to show that this measurement considered alone was of any practical value in assessing the potential fertility of semen samples for use in routine insemination work. Cummings (1954) found no change in conception rate with initial spermatozoal concentrations ranging from 2.5 to 19 x 10^8 per ml. although the higher counts appeared to result in higher fertility; variations in the counts were also reflected in the measurement of respiration and impedance change frequency. Erb, Flerchinger, Ehlers and Mikota (1955,a) reported that a variation in spermatozoal concentration was the main factor interfering with metabolism tests.

However, the measurement of spermatozoal density is required when high semen dilution rates (over 1:100) are used in order to ensure that the spermatozoal content of the diluted semen remains above the threshold level required for optimum fertility (see page 151).

(ii) Initial motility assessment. Although widely used in insemination practice, initial motility assessment is a subjective measurement and the results obtained by different workers cannot be reliably compared. Both this assessment and the estimation of the motility of stored semen can be used to detect gross differences in semen quality, but not small fertility differences. The practice is to rate the samples by the character of the wave observed in a fresh smear of uniform thickness under a standard size of coverslip at 37°C. The character of the wave is dependent both on the activity of the individual spermatozoa and on their concentration.

In a detailed paper on the theory of wave motion Walton (1952) showed that when spermatozoa in suspension were caused to lie parallel to one another the suspension allowed more light to pass through it than when the spermatozoa were not orientated. He thought that the explanations of wave motion put forward previously, that is that the waves represented different concentrations of spermatozoa (Rothschild, 1948) and that the dead spermatozoa were pushed together in bands of the live ones (Blom, 1946), were not satisfactory. It was his opinion that the light and dark areas, or waves, seen in semen, were the result of different streams of spermatozoa, all the spermatozoa within a stream being orientated relative to one another and

scattering the light to different degrees. In the semen the forces tending to cause orientation are balanced by the disorientation produced by the streams moving in all different directions; however, when a thin film of semen is prepared as, for example, under a coverslip, the speed of movement of these streams, comprised of actively motile spermatozoa, creates local disturbances of equilibrium in the medium and wave motion results. In Walton's view the segregation of the dead cells is a consequence of wave motion, as spermatozoa with equal velocity will tend to come together, and not a primary cause of it. Methods of scoring the motility of semen samples have been given by several workers (Herman and Swanson, 1941; Swanson and Herman, 1944; Emmens, 1947). Blom, (1946) devised a comparator chamber, in which semen smears of different thickness could be examined simultaneously, thus permitting a quick assessment of the wave motion in the deeper part and of the activity of the individual spermatozoa in the shallower part of the slide. Cummings (1954) advised the use of phase contrast microscopy when making motility assessments.

Amongst bulls at a centre, there is no clear evidence that initial spermatozoal motility can be closely correlated with fertility (Swanson and Herman, 1944; Lasley and Bogart, 1943; Cheng, Casida and Barrett, 1949; Cupps et al. 1953). Erb, Ehlers, Mikota and Schwarz (1950), Stone, Johnston and Mixner (1950) and Bishop et al. (1954) found initial motility to be more closely correlated with other semen characters than with fertility. Since an examination of smears of undiluted semen of high density can only give an approximate

indication of the numbers of actively motile spermatozoa, there would appear to be some justification for examining a preliminary low dilution of the semen in order to make a more accurate assessment of the degree of motility. Such a procedure was described by Willett and Salisbury (1942). However, objective methods of determining motility of semen are being developed and these may permit more critical assessment of this characteristic in relation to fertility to be made (see page 77). Nevertheless, there is clear evidence that motility does not necessarily indicate that spermatozoa are capable of fertilisation. Mann (1958) obtained a normal fructose utilisation rate with semen from an infertile Guernsey bull (see page 57). In the semen of this bull, the heads of the spermatozoa were completely detached from the mid-piece and tail, but the latter were motile, and shown to be capable of utilising fructose.

It is usual in routine insemination practice to record the ejaculate volume simultaneously with the initial motility. From the literature cited by Anderson (1945) the average volume of an ejaculate appeared to be 4 ml. There were indications that the volume produced varied with the breed and age of the bull but no relationship between this and fertility was found. Blom (1950a) reported that ejaculate volume was reduced in bulls which were practically sterile. Although Bishop et al. (1954) found evidence of a decline in fertility with increasing ejaculate volume and increasing age, selection by them against ejaculates of low volume may have been

responsible for this finding.

(iii) Assessment of live/dead spermatozoa by the differential staining technique. Differential staining was first used for spermatozoa by Lasley, Easley and McKenzie (1942) who, by using eosin and opal blue stain in an isotonic phosphate buffer, showed that the dead spermatozoa, which stained with eosin, could be readily distinguished from the living unstained spermatozoa, against the background stained with opal blue. Mayer, Squiers and Bogart (1947) and Mayer, Squiers, Bogart and Oloufa (1951) claimed that fast green (F.C.F.) was superior to opal blue as a background stain, while Shaffer and Almquist (1948) preferred 4 per cent aniline blue. Blom (1950,b) found it preferable to use a water soluble nigrosin as a background stain along with the eosin. Hancock (1951), who recommended 30 g. aqueous nigrosin (G.T. Gurr), 5 g. eosin Y. water soluble (G.T. Gurr) in 300 ml. distilled water, drew attention to the necessity of having the stain mixture and semen at a uniform temperature (30°C. was recommended), in order to avoid increasing the number of dead spermatozoa through mixing semen and stain of different temperatures. It was later reported (Bishop et al., 1954) that this nigrosin/eosin solution was hypotonic to bull semen and had a pH value of 8.5. Swanson and Bearden (1951) found that, when Blom's method was compared with other treatments, there was a significantly higher percentage of dead spermatozoa and the difference was shown to be due to the use of a hypotonic solution. These authors found that 1 per cent eosin.B. and 5 per cent nigrosin in isotonic citrate buffer gave constant results even when the proportion

of semen to stain varied from 1:1 to 1:20 and the pH value from 6.4 to 8.7. This finding was in contrast to the results reported by Lasley et al. (1942) and by Mayer et al. (1951) who had stressed the importance of having the mixture at a constant pH value of 6.8.

The repeatability of estimations made by this technique has been studied by various authors. Ortavant, Dupont, Pauthe and Roussell (1952), using a 4 per cent aniline blue and 1 per cent eosin in a phosphate buffer pH value 6.78, which was described by Shaffer and Almquist (1948), found no differences in the percentages of dead spermatozoa in the same semen counted by two operators. Furthermore, the results obtained by counting 150 spermatozoa per smear were not appreciably different from those obtained when 2,000 spermatozoa were counted. However, a significantly lower value for the number of dead spermatozoa was obtained when a diagonal section of the smear was examined, indicating an uneven distribution of cells in the smears. Campbell, Hancock and Rothschild (1953), reporting on the counts made separately by two operators, found that the variation between different counts on one smear was consistent with random sampling from a binomial population. The same applied to variations between slides, made from one sub-sample, but not to variations between sub-samples. The number of spermatozoa, that these workers considered should be counted in order that a reliable estimate might be obtained, varied according to both the numbers of dead spermatozoa counted and to the numbers of sub-samples examined from the original ejaculate. These findings were not confirmed in subsequent work from that laboratory. Campbell, Dott and Glover (1956),

using bull, boar and ram spermatozoa, found larger variations between counts of the same semen sample than would be expected if the distribution of the stained spermatozoa was uniform. It was suggested that this was due partly to the "clumping" of dead spermatozoa and partly to the occurrence of half stained forms, which might be classified as "live" by one operator and "dead" by another. Species differences in these variations were also recorded, the incidence of clumping being low in the bull and boar samples in this study. The occurrence of half stained forms had been reported by Mayer, et al. (1951) and by Brochart and Debatène (1953); Brochart (1954) associated this with the degree of maturity of the spermatozoa, those from the caput of the epididymis showing a greater permeability to eosin than those from the middle portion and the tail of the epididymis. Campbell et al. (1956), as a result of finding that the degree of staining was less when distilled water was used in place of sodium citrate as the stain solvent, postulated that the degree of staining was due to variations in tonicity, but they did not study the effect of an isotonic stain solution, Dott (1956), using hypo- and hyper-tonic nigrosin-eosin solutions with pH values ranging from 6 to 8, reported that the spermatozoa stained differently in stains with different pH values and also that, when the stain was dissolved in water in place of citrate solution, fewer spermatozoa were stained. Campbell et al. (1956) emphasised that the time between preparation of the sub-sample and the making of the smears should be kept constant, five minutes being considered satisfactory. The use of a citrate diluent as a solvent for the stain in order to reduce the numbers

of partially stained forms, the selection of the fields for counting without avoiding clumps of spermatozoa and the adoption of a definite criterion for stained and unstained forms were also advised by these authors. From their data it would appear that the maximum accuracy should be obtained by preparing one smear from each of several sub-samples and by counting 100 spermatozoa on each smear. In this work it was shown that with a smear count of dead spermatozoa of 20 per cent, obtained by counting a total of 400 cells (100 from one smear from each of 4 sub-samples), the approximate variation in this would be 20 $\pm$ 6.0, i.e. 14 to 26 per cent whereas, with a total count of 200 cells on one smear from each of two sub-samples, this range would be 20 $\pm$ 8.4, i.e. 11.6 to 28.4 per cent.

A high incidence of spermatozoa with bent tails in smears stained by a hypotonic nigrosin/eosin solution was reported by Bishop et al. (1954) (see page 59), but no reference was made to the finding of half stained forms in their extensive study. A further modification of the nigrosin/eosin mixture to give a solution isotonic with the semen has been described by Hancock (1957). It requires careful preparation to avoid contamination since it contains glucose and it must be stored in a refrigerator. The fact that this staining technique does not differentiate motile from non-motile spermatozoa but living from dead spermatozoa was pointed out by Lasley (1951), who found a highly significant correlation between the percentages of live, (i.e. unstained) motile and progressively motile spermatozoa in fresh semen, the last two characteristics being measured by a haemocytometric method.

Bane (1952) found only small differences between the percentage of unstained spermatozoa and that of motile spermatozoa, as estimated by a haemocytometer slide.

Other findings, which may lead to alternative methods of assessing the number of dead spermatozoa, have been reported. Hancock and Shaw (1955) demonstrated that only the dead spermatozoa agglutinated when a semen sample was treated with a formalin diluent. Bangham and Hancock (1955) showed that the opacity of a semen suspension could be altered by filtration through a medium of glass beads which removed the dead spermatozoa. The change in opacity was found to be significantly related to the percentage of stained spermatozoa in the semen samples tested ($r = 0.7$, $p < 0.001$). A further alternative staining procedure was reported by Bishop and Smiles (1957) who, by using ultra-violet light, dark ground illumination along with premulin stain, found that dead spermatozoa fluoresce light blue and the living ones remain invisible.

Relationship between percentage of dead spermatozoa and fertility. Lasley et al. (1942) and Lasley and Bogart (1943), working with data from insemination of beef cattle, found that semen samples containing less than 50 per cent live spermatozoa were of doubtful fertility. Madden, Herman and Berousek (1947) and Stone et al. (1950), who used a similar staining technique to Lasley and his co-workers, could not confirm this. However, these observations were based on relatively small numbers of inseminations. Other workers (Erb et al., 1950; Cupps et al., 1953)

found a live spermatozoal percentage to fertility relationship that did not appear to be of any great practical value in estimating potential fertility. In an extensive study with bulls in routine insemination use, Bishop et al. (1954) found a mean of 22.1 per cent dead spermatozoa in 122 samples tested and, although unable to find a correlation between numbers of living spermatozoa per insemination and fertility, they did demonstrate a significant relationship between the percentage of dead spermatozoa in the ejaculate, impedence change frequency (I.C.F.) and fertility. They suggested that this indicated that fertility level was determined by qualitative differences in the living spermatozoa rather than by a reduction in their numbers. However, it must be pointed out that in this survey the variations in the percentage of dead spermatozoa were found to account for only 17 per cent of the conception rate variations in the bulls tested (see page 80).

This conception rate relationship was found to be significant in the bulls at only one of the five centres visited. In a subsequent but more limited study, Campbell, Hancock and Shaw (1960) were unable to demonstrate any correlation between the frequency of dead spermatozoa (average in the study was 21.0 per cent) and fertility. They pointed out that the previous findings of Bishop et al. (1954) were largely due to the low conception rate (38.9 per cent) of one bull.

(iv) Count of abnormal spermatozoa. The published work on the preparation, staining and examinations of semen smears for abnormal spermatozoa was reviewed by Anderson (1945). In the early work, Williams and Savage (1925), who studied 208 bulls, and Lagerlof (1934), who

studied 50 bulls, found lowered fertility when more than 17 per cent of abnormal spermatozoa was present; these workers attached most importance to the morphology of the spermatozoal head, the main abnormalities being either its complete detachment or abnormal shape. Similar results were reported by Davis, Trimberger and Underbjerg (1940,a) and by Anderson (1941). Although Herman and Swanson (1941) concluded that the presence of up to 30 per cent abnormal spermatozoa was compatible with either good or poor fertility, these authors claimed that this high abnormality rate was due to the inclusion of tail defects which were unaccompanied by abnormal heads; however, as pointed by Anderson (1945) other workers had also taken note of this coiled tail defect and probably Herman and Swanson had used a different classification of abnormal forms. Subsequently Laing (1945,a), after examining 3 or 4 ejaculates from each of 14 bulls of different fertility, was unable to demonstrate any relationship between the incidence of abnormal spermatozoa and conception rate in natural mating. He indicated that, in spite of using a similar classification of abnormal forms, his different results could be due to the use of a different method of collection and examination of the semen than that adopted by Williams and Savage and by Lagerlof, who apparently obtained the semen from the vagina of a cow served by the bull. Laing (1945,a) who collected the semen by an artificial vagina and examined the spermatozoa fresh and unstained in normal saline, also pointed out that artefacts, which could be produced in the fixing and staining, and differences in the criteria of fertility, could account for the different results. However, with the use of fresh unstained and unfixed

smears it would appear to be more difficult both to protect the semen against temperature shock and also to examine and classify the types of spermatozoa present. Rollinson (1951,a), in a study of 29 infertile or sterile bulls and 14 fertile bulls used in natural mating, found that the incidence of abnormal spermatozoal forms was not closely correlated with fertility. His results indicated that, with an incidence of approximately 4 per cent abnormal heads, 5 per cent detached heads, 10 per cent deformed mid-piece and 1 per cent abnormal tails, a fertility range of 40 per cent to 60 per cent or 2.5 to 1.7 services per conception could be expected with such a bull. The extent to which the findings in bulls used for natural service are applicable to insemination bulls is doubtful but there have been few recent critical studies of this at insemination centres. Blom (1948) classified the spermatozoal abnormalities as (a) primary due to defective spermatogenesis (i.e. imperfectly formed spermatozoa, small and possibly detached heads, double tails and immature forms) and (b) secondary as a result of degenerative changes following a normal differentiation of the spermatozoa (these included normal but detached heads, proximal and distal protoplasmic droplets, bent tails and detachment of the galea capitis); when more than 15 per cent of the spermatozoa showed a primary abnormality there was impaired fertility accompanied in many instances by testis degeneration or hypoplasia. In a study of 100 normal fertile insemination bulls, Blom (1948) found that approximately 5 to 10 per cent of the spermatozoa showed a primary abnormality but the incidence of secondary abnormalities appeared to vary somewhat. Although some of these (i.e.

detached heads and bent tails) could be artefacts they could also be a sign of testis degeneration and therefore be an indication of impaired fertility. However, it should be appreciated that an examination of the fresh semen under a coverslip should reveal if this is a true abnormality or an artefact. The presence of over 3 per cent of spermatozoa with proximal protoplasmic droplet was also considered to be of some significance. In a similar study of 76 bulls at insemination centres, Bishop et al. (1954) reported a mean incidence of abnormal forms, excluding bent tails, of 6.1 per cent. In this work the classifications were slightly different, the primary forms included the cytoplasmic droplets attached at the neck, the secondary forms did not include the spermatozoal tail deformities which were separately classed as tertiary forms. The mean incidence of each of these forms was 4.3 per cent, 1.8 per cent and 19.5 per cent respectively, but the high incidence of the last classification was considered to be due to an artefact. In the detailed analysis of their data, Bishop et al. (1954) were unable to demonstrate any relationship between the incidence of abnormal spermatozoa and fertility but the incidence of abnormal forms was uniformly low, possibly because of the narrow range of fertility of the bulls in this study. Cupps, et al. (1953) reported a high correlation between percentage of abnormal spermatozoa and fertility but, when interpreting their results, it should be borne in mind that the low fertility may have been due to a recessive gene. Although Rottensten and Andersen (1956) found that one low fertility bull in their study had an average abnormality rate of 30 per cent, from their overall results they concluded that a high level of

abnormal spermatozoa was not necessarily indicative of low fertility.

The published reports show that morphological examination of the semen is of limited value in the assessment of fertility, but this should still be carried out since there are definite spermatozoal abnormalities associated with complete infertility. The now widely recognized acrosome defect of Friesian bulls was referred to by Blom (1948), Hancock (1949), Rollinson and Makinson (1949), Teunissen (1946). Assessment of the incidence of this spermatozoan defect is best done on Giemsa or Indian ink stained smears. It is not readily noticeable with the commonly used nigrosin-eosin stain once the spermatozoa are dead owing to a change in the acrosome (Hancock, 1953). Presumably this explains the report by Rollinson and Makinson (1949) that the incidence of this abnormality decreases from around 42 per cent soon after collection to 30 per cent 24 hours later and to 10 per cent after 96 hours storage. A detached head defect has been found to be associated with almost complete infertility in Guernsey bulls by Haq (1949) and Hancock and Rollinson (1949). Since with both these conditions the spermatozoa are living and motile, the defect could be overlooked if no morphological examinations were made. Hancock (1955) showed that the separation of the heads and tails of the spermatozoa actually occurred in the caput epididymis and was associated with the migration of the cytoplasmic droplet. Blom (1959) reported what is referred to as a cork-screw type of mid-piece defect, which appears to be associated with reduced fertility. The addition of seminal vesicular secretion to semen from the

epididymis has been reported by Bialy and Smith (1958,a) to result in a marked reduction in the number of protoplasmic droplets. Although Hancock (1952) indicated that the fragile acrosome cap, which normally is closely applied to the surface of the spermatozoal head, could become detached under certain circumstances to give rise to what Blom (1945) had called the galea capitis, Wu and Mackenzie (1955), following studies with the electron microscope, claimed that the acrosome cap and galea capitis were in fact two different structures; more recently Karras (1958) indicated that the galea capitis consisted of an inner and outer cap.

As indicated above it is important that, in the collection, handling, smearing and staining of the semen, care is taken to avoid damaging the spermatozoa, and thereby increasing the abnormality rate. Mercier and Salisbury (1947) advised that fewer artefacts would be produced by making thin semen films and staining these uncleared. Hancock (1952), after finding that the so called galea capitis occurred only in dead cells, pointed out that many of the dead spermatozoa could have been killed by temperature shock before fixation, and emphasised the need to avoid the risk of damage to the spermatozoa.

Morris (1950) showed that with a phase contrast microscope the less evident changes in the spermatozoa could be more easily detected and this obviated the need for staining the smears, thereby reducing the risk of producing artefacts.

More recently, Walton (1957) demonstrated, by means of electron micrographs, that the outline of the head of ram spermatozoa alive at the time of fixation differed markedly from the heads of those that were either dead or subjected to cold shock before fixation, although in the latter case disentegration of the cell surface was more marked than in the former case. It therefore appears that, by using such a method, a detailed study of the spermatozoa for evidence of damage could be reliably carried out, both for the assessment of semen quality and for determining the effect of, for example, handling and deep freezing on semen.

Bishop et al. (1954) reported that the high incidence of spermatozoa with bent tails in one series of their observations was probably the result of suspending the spermatozoa in a hypotonic solution (nigrosin/eosin), even although precautions were taken against temperature shock. However, when they mixed warm semen with the hypotonic cold nigrosin/eosin stain the proportion of bent tails was reduced, indicating that the adverse effect of hypotonic solutions was most marked with semen which had been protected against cold shock. Previously Pursley and Herman (1950) reported that dilution in a hypotonic media increased the numbers of spermatozoa with abnormal tails. Although Salisbury, Willett and Seligman (1942) showed an increase in this abnormality in cooled semen, Morris (1950) claimed that the incidence of bent tails was decreased if the semen was cooled rapidly. It is possible that the effect of this interaction between cooling and osmotic

tension on the incidence of bent tails in spermatozoa may have been overlooked by earlier workers.

The reproducability of the counts of abnormal spermatozoa must also be considered. Although Salisbury and Mercier (1945) concluded that a count of 100 spermatozoa was as reliable as the examination of 500 spermatozoa on each of 2 slides from one ejaculate, there would appear to be some merit in examining more than one slide in order to detect artefacts. Bishop et al. (1954) counted 400 on one slide to assess the incidence of abnormal forms.

Recently Campbell, etal. (1960), in an attempt to reduce the variations in the morphological characteristics of the sample between its collection and examination, concluded that the dilution of the semen in buffered formal agar immediately after collection and the subsequent examination of a wet fixed preparation at 37°C. was the most reliable procedure. (This confirmed the early work of Laing (1945,a)). By this method in their study of 257 ejaculates from 13 bulls the average (per cent) incidence of the abnormalities were:- Malformed heads 0.98, malformed mid-pieces 0.89, bent tails 4.56, coiled tails 0.43, detached heads 2.63, detached tails 1.85, neck cytoplasmic beads 0.93, mid-piece cytoplasmic beads 6.83. The overall 90 day non-return conception rate from 3,744 inseminations with the samples studied was 71.6 per cent (in only 2 bulls was this less than 67 per cent). Although the overall abnormality counts were low, the counts of cytoplasmic droplets and of bent tails varied between samples within bulls and contrasted with the counts of deformed heads

and mid-piece defects, which were more constant and, therefore, characteristic of a bull. Although this study covered a limited bull population of above average fertility, there was no evidence of a lowering of fertility with an increase in the frequency of abnormal or detached heads or of dead spermatozoa, but there was a negative correlation (significant at the 5 per cent level) between fertility and incidence of neck cytoplasmic beads.

B. Biochemical Tests

Although the metabolism of semen was considered to be predominantly glycolytic (Mann, 1949), its respiratory mechanism and its dehydrogenase activity have also been extensively studied with a view to finding a correlation between the metabolism and the fertilizing capacity of semen. The following aspects have been investigated:-

(i) Change in pH value. Measurements of the pH value of semen at the time of collection have not been shown to be of any practical value. Anderson (1945) reported that semen of poor quality was generally neutral or slightly alkaline but the semen of fertile bulls had usually a pH value of less than 7.0. The range could be quite wide and this measurement alone was of little value. However, Anderson (1945, 1952) showed that between bulls there were highly significant differences in the change in pH value occurring in semen samples during incubation. Laing (1945,a), Reid, Ward and Salsbury (1948), Romijn (1948) and Fiser (1952) confirmed that the degree of fall in pH value of semen on incubation gave an indication of semen quality. However, for the results of this

test to be comparable, it must be carried out at 37°C, under anaerobic or partially anaerobic conditions. The change in pH value is also dependent on such characteristics of the sample as spermatozoal concentration and fructose content. Mann (1954) indicated that this test was of limited value as a measure of the metabolic changes in a sample, since a fall in pH value reduced motility and metabolic changes in the sample. Erb et al. (1950) and Buckner, Willett and Bayley (1954) could find no correlation between a fall in pH value during incubation and fertility.

(ii) Methylene blue reduction time. The time taken for a standard solution of methylene blue to be decolourised under standard conditions of incubation by a sample of semen is said to give an indication of the dehydrogenase activity of the sample. The test, a modification of Thunberg's dehydrogenation test, was originally described by Sørensen (1942). The reduction time of the methylene blue by semen in a gelatine diluting fluid was determined. Alternative methods were described by Milovanov and Sokolovskaya (1943) and by Brochart (1948), who measured the decolourisation time in a methylene blue/semen mixture in the central position of a capillary tube, and by Beck and Salisbury (1943), who used small diameter precipitin tubes, the surface of the semen/methylene blue mixture being covered by a layer of liquid paraffin after mixing. In view of the different methods adopted for this test, and also since the rate of decolourisation can be influenced by substances

such as glucose, citrate and lactate that are present in the semen or diluent (Lardy and Phillips, 1941), one is not able to compare the reduction times given by different workers.

The methylene blue test is rendered of little value by the dependence on other semen characteristics that the reduction time displays. Bishop et al. (1954) with 112 observations on 76 bulls had an average reduction time of 7.5 minutes when semen was diluted in yolk-phosphate buffer. In samples shown to have insufficient fructose the average time was found to be only 3.3 minutes whereas in the presence of sufficient fructose it was 6.6 minutes. The maximum time of 9 minutes laid down by Branton, James, Patrick and Newsom (1951) was determined by a modified technique in which the semen was diluted to contain 12-15 million motile spermatozoa per ml. Whilst most workers agree that the reduction time is directly related both to motility and to spermatozoal density, and inversely to the number of dead spermatozoa, there is little evidence of any useful relationship with fertility (Erb et al., 1950; Buckner et al., 1954; and Bishop et al. 1954). However, Jenichen, Schmidt, and Strassburg (1956) have demonstrated a significant correlation between methylene blue reduction time and conception rate of semen used for nominated insemination after 4 days storage, but this would require to be investigated further with large numbers of inseminations.

(iii) Resazurin test. The use of the resazurin test for semen quality assessment has been investigated by Erb and Ehlers (1950)

and by Erb, Ehlers and Flerchinger (1952). These workers used a constant number of spermatozoa in a phosphate buffer and found that the reduction times required for the colour to change, firstly to pink and secondly to white, were significantly related to the methylene blue reduction times. Although a correlation between resazurin reduction time and fertility was demonstrated by these authors, this accounted for only a small part (10 per cent) of the variations between bulls and between samples. A further study by Erb, Flerchinger, Ehlers and Mikota (1955,a) showed that in a citrate buffer an average reduction time of 5.7 minutes was obtained. This compared with 5.2 minutes when phosphate buffer was used. High correlations were obtained with other measures of metabolic activity, but no fertility data were included in this study and, in view of the influence of spermatozoal concentration, these authors found difficulty in the interpretation of their results with this test.

(iv) Oxygen uptake. The respiration of semen, as measured manometrically, has been correlated with motility and concentration of the spermatozoa. Walton and Edwards (1938) reported a positive correlation between the O_2 uptake of semen and its fertility in natural service. However, Ghosh, Casida and Lardy (1949) and Bishop et al. (1954) were unable to confirm this with semen used for artificial insemination.

Oxygen is utilised by whole semen partly for its exogenous respiration, (i.e. oxidation of extra-cellular substrates, the most

important being the lactic acid produced by fructolysis, and partly for the endogenous respiration (i.e. oxidation of intra-cellular material as occurs with epididymal spermatozoa). The measurement of oxygen uptake is associated with metabolic changes occurring in whole semen. After washing spermatozoa clear of seminal plasma by centrifuging and resuspending in Krebs calcium-free phosphate buffer, the respiration (i.e. endogenous) is often considerably reduced when compared with that of the whole semen, (Lardy and Phillips, 1943). Ghosh et al. (1949) found some bulls to have exceptionally low values for the endogenous respiration, but Melrose and Terner (1953) found that, although this varied between bulls, it was relatively constant within bulls and the addition of fluoride (0.02 M.) appeared to bring the endogenous respiration of all bulls down to a more uniform low level. Lactate, pyruvate, acetate and possibly other organic compounds can be oxidised by bull spermatozoa. It has been suggested by Lardy and Phillips (1945) that the oxidative changes probably take place through the Krebs tri carboxylic acid cycle. However, the pattern of the endogenous oxidative metabolism may require further study since plasmalogen, and not the phospholipid lecithin, is now considered to be involved (Hartree and Mann, 1958). It should also be noted that Bomstein and Steberl (1957) reported that neither ejaculated nor epididymal spermatozoa could oxidise either exogenous or cellular phospholipid to any extent. Although respiration is considered to be associated with motility, Mann and Lutwak-Mann (1948) showed that, under experimental conditions, the motility and fructolysis of semen could be abolished without greatly suppressing respiration.

Tosic and Walton (1950) demonstrated that in the standard yolk diluents certain toxic end products could sometimes be formed with a resultant depressant action on spermatozoal respiration; however, Bishop and Salisbury (1955,b) showed that O_2 uptake actually improved in the presence of a yolk-saline-phosphate buffers when compared with that of undiluted semen.

These studies showed how difficult it is to assess the significance of oxygen uptake measurements. In view of the availability of alternate metabolic pathways, the exact significance of oxygen uptake measurements of whole semen cannot always be interpreted unless there are simultaneous estimations of the substrates utilised. This was revealed in pyruvate metabolism studies by Melrose and Terner (1953). These workers were unable to demonstrate the precise effect of 2:4 dinitrophenol on semen until the actual amount of pyruvate used up was estimated simultaneously with the manometric measurement of the oxygen uptake.

(v) Fructolysis. Redenz (1933) discovered that reducing sugars could be utilised by spermatozoa for maintaining their motility under anaerobic conditions, and Comstock and Green (1939) concluded that the measurement of glycolysis was the best criterion for predicting the potential fertility of fresh semen. However, Mann (1946,ab) demonstrated that the reducing sugar present in bull seminal plasma was fructose and not glucose, the concentration varying between 280 and 700 mg/100 ml. (Mann, 1954) and its secretion being controlled by the testicular hormone testosterone (Mann, Davies and Humphrey, 1949). Mann (1948) described a method of semen quality assessment in which the rate of fructose

utilization in semen samples was measured under anaerobic conditions. This test was designed for use at insemination centres. It needed the minimum of laboratory equipment and less than 0.5 ml. semen. Various workers have investigated the use of this test with rather conflicting results. Gassner and Hill (1952), Bonadonna and Pozzi (1954), Probine, Mc.Cabe and Shannon (1958) and Schmidt and Steger (1957) found a definite relationship between fructolysis and fertility, but Rollinson (1951,b), using infertile bulls, reported inconclusive results. Melrose (1952,a) was able to correlate fructolysis with fertility in only 3 of the 8 bulls tested; his findings were in accord with those of Cupps et al. (1953) and of Ehlers, Flerchinger and Erb (1953). Although Bishop et al. (1954) found a significant direct relationship between the amount of fructose utilised per ml. semen and fertility, but not between fructose utilisation per living spermatozoon and fertility, a highly significant direct relationship existed between fructolysis per ml. semen and the concentration of living spermatozoa; fructolysis per living spermatozoon decreased with increasing concentration of living spermatozoa and increased with increasing concentration of fructose.

Most studies have revealed wide variations in the initial concentration of fructose, the possible effect of which, together with the effect of the spermatozoal concentration on fructolysis, focussed attention on the need for studies of the basis of assessment of fructolysis and also of the effect on it of other seminal characteristics. The "fructolysis index"(Mann, 1948) was defined as the amount of fructose

utilised by 10^9 spermatozoa over one hour; for bull semen it was recommended that this measurement should be made at hourly intervals over a three hour incubation period. However, in field studies (Melrose, 1952,a; Bishop et al., 1954) it was revealed that with the semen of certain bulls the initial level of fructose was so low that it was all used up within the first hour. Obviously fructolysis could not be reliably measured on an hourly basis in such semen. Although Comstock and Green (1939), Erb, Flerchinger, Ehlers and Gassner (1956) and Hopwood Rutherford and Gassner (1956) pointed out that the concentration and motility of spermatozoa were both related to the rate of utilization of the fructose in the seminal plasma, Salisbury and Van Demark (1945) claimed that fructolysis was also related to the level of carbohydrate present. The effect of the spermatozoal concentration was noted by Hopwood et al. (1956), who also reported that the rate of decline in fructolysis over a three hour period was of some importance. Erb et al. (1956), using previously published data, calculated the fructolysis utilisation rate over a 10 minute period and found that it was directly proportional to the number of spermatozoa in the sample incubated. Fructolysis, when estimated after a ten minute incubation period, was found to be more closely correlated with fertility than was the rate of utilisation obtained over the hitherto conventional period of 60 minutes. An incubation time of 20 minutes at 37°C. was suggested for semen of high spermatozoal count and good motility.

Mixner, Mather and Freund (1957) claimed that the rate of fructolysis, as measured by Mann (1948), could be considered in two separate parts:- (a) the "Fructolysis Coefficient", which was defined as the fractional decline in fructose utilisation measured over a period of up to 60 minutes; and (b) the "Maximum Fructose Utilisation", which was the amount of fructose utilised by 10^9 spermatozoa in 1 minute at the start of incubation. They considered that (a) would be independent of the initial level of fructose and the time between collection of semen and start of incubation, while (b) would be influenced by these factors. Both (a) and (b) were thought to be independent of spermatozoal concentration. Although the "Maximum Fructose Utilisation", calculated after incubation for one minute, should theoretically be preferable to the 10 minutes incubation period reported above by Erb et al. (1956), Mixner et al. (1957) pointed out that in practice the most accurate estimations could be made after longer incubation periods provided that the fructose supply did not become exhausted. By making fructose estimations after incubation for 20 minutes, 40 minutes and 60 minutes at 37°C., Freund, Mixner and Mather (1957) showed that, whereas the higher initial fructose levels and higher spermatozoal concentrations were associated with higher fructose utilisation rates, these factors did not influence the decline in rate of fructolysis (i.e. the fructolysis coefficient referred to above.). The authors suggested that fructolysis could be assessed either by the fructolysis coefficient or by applying a correction factor to the fructose utilisation rate to allow for variation in initial fructose and spermatozoal concentration. Freund,

Mixner, and Mather (1959,a) were unable to ascertain the reason for increased fructolysis following the addition of fructose, but they confirmed the above findings, and also showed that fructose addition increased its utilisation even in those samples with low spermatozoal concentration and high initial fructose levels. They suggested also that fructose could usefully be added as a routine to all incubated semen samples. Although the application of these findings to the assessment of fertility has not been investigated, Freund and Murphree (1959) repeated the above studies with 262 semen samples from 10 bulls showing wide variations in semen characteristics. They confirmed again that differences in fructose utilisation could be accounted for by variation in spermatozoal counts, initial fructose level and initial motility of the sample. It was emphasised that, in attempting to relate fructolysis to fertility, corrections must either be made for these factors, or else the measurement of fructolysis should be carried out in a manner which had been shown to be independent of these variables. However, the fact that it has not been possible to determine what between bull variations in fructolysis, independent of spermatozoal concentration, initial fructose level and motility, can occur, suggests that fructolysis is of limited value in semen quality assessment.

Vantienhoven, Salisbury, Van Demark and Hansen (1952) showed that spermatozoa utilised glucose in preference to fructose. It should be noted that because of this the fructolysis test cannot be performed in the presence of egg yolk, which contains glucose. The fructolysis

coefficient was found to be unaffected by changes in the temperature or duration of incubation (Freund, Mixner and Mather, 1959,b), although these factors did alter the rate of fructose utilisation. When the incubation period was extended to 6 hours, it was found that fructose utilisation and lactic acid formation continued after motility had ceased (Erb, Albright and Ehlers, 1959).

Freund and Mixner (1959) determined what effects certain hormones and amino-acids had on fructolysis. Epinephrine inhibited fructolysis, but insulin, thyroxin, tri-iodothyronine, ergothionine, and cysteine had no effect. Nakabayashi and Salisbury (1956) reported insignificant variations in fructolysis with semen collected at different seasons of the year and, subsequently, these authors (1959) indicated that these differences could not account for any seasonal variations in fertility.

Whilst fructolysis would appear to give some measure of the metabolic changes in a semen sample, the reports on its usefulness for assessment of potential fertility are conflicting. Some variations in these results could undoubtedly be accounted for by the influence of other factors, such as spermatozoal density and initial fructose level, which are known to affect fructolysis, but alternative methods of calculating the latter may help in this direction. In particular, the fructose coefficient (i.e. rate of falling off in fructose utilisation) would appear to be worthy of study in relation to fertility, and it has been suggested that this would be useful for estimating semen quality in laboratory investigations. However, a high degree of accuracy is essential when measuring fructose levels after short incubation

intervals, and this could preclude its use under normal insemination centre conditions. Hitherto attention had been concentrated on the anaerobic utilization of fructose and other compounds by spermatozoa, but it is now appreciated that, in vivo, the aerobic metabolism of spermatozoa is of importance (Bishop D.W., 1956). The possible effects of other unrecognised biochemical reactions occurring in the semen concurrently with fructolysis cannot be overlooked when working with whole semen.

(vi) Pyruvate utilisation. In studies of washed spermatozoal suspensions, to which fluoride was added to reduce the endogenous metabolism to a low level, Melrose and Terner (1952,1953) reported that the quality of the semen samples tested could be graded according to the oxygen consumption after the addition of pyruvate and pyruvate plus 2:4 dinitrophenol. They reported that, with washed spermatozoa from highly fertile bulls under the above conditions, the oxygen uptake, which was low in the presence of pyruvate plus fluoride, was increased two-fold or more with the addition of 2:4 dinitrophenol. The semen from low fertility bulls showed a relatively higher oxygen uptake with pyruvate and fluoride and no marked further increase on addition of the 2:4 dinitrophenol. These results were obtained at one centre with 60 semen samples from 16 bulls (3 had 112 day non-return conception rates of over 65 per cent, 8 were within the 55 to 65 per cent range and 5 less than 55 per cent); a more extensive study by Glew (1956,a and b) covering 291 semen samples from 74 bulls at 8 centres confirmed the above findings. Glew (1956,b)

pointed out that the semen from a particular bull should be tested on atleast four different occasions before a decision could be made on the metabolic pattern of the semen under the above system. This test entails the use of manometric equipment for the measurement of oxygen uptake and is therefore somewhat difficult to carry out under routine insemination conditions.

(vii) Hyaluronidase content. Johnston, Stone and Mixner (1949) reported that the significantly different levels of hyaluronidase between individual bulls depended on the spermatozoal count of the samples tested. Subsequently Johnston and Mixner (1950) found no significant relationship between hyaluronidase titre and fertility with semen diluted up to 1:100. Although Sallman and Birkeland (1948) had previously found with semen diluted 1:55 in yolk-citrate that there was a significant relationship between hyaluronidase level and fertility, they also indicated that fertility was reduced above a certain threshold level of hyaluronidase. Jacquet (1952) estimated the hyaluronidase concentration by intradermal injection of semen along with India ink into guinea pigs and then compared the area of its subsequent dispersal with that produced by India ink alone; by this method a positive relationship with fertility was claimed. The effect of the actual spermatozoal concentration and dilution rates would require to be taken into consideration in attempts to relate hyaluronidase levels with fertility.

(viii) Other biochemical changes. An attempt by Flerchinger, Erb, Mikota and Ehlers (1956,b) to correlate lactic acid accumulation with

fertility failed. Flerchinger and Erb (1955) were unable to demonstrate any relationship between inorganic and total phosphorus in semen and fertility. Blom (1955) reported on the use of the catalase test as a measure of the degree of bacterial contamination of semen samples, but this has not been extensively studied. Measurements of the reduction time and Vitamin C content were reported by Crespo and Solana Alonso (1958) to be of no value in semen quality assessment. Citric acid is present in relatively high concentrations in bull semen (510 - 1100 mg per 100 ml.) and, as with fructose, its secretion is controlled by the male sex hormone testosterone, (Humphrey and Mann, 1948). Lardy and Phillips (1945) showed that citric acid could be utilised by the spermatozoa but Humphrey and Mann (1949), although not confirming this, could not show that it was beneficial for either aerobic or anaerobic metabolism, and there was no indication that citric acid was an important source of nutrient for the spermatozoa. Mann (1954) indicated that citric acid may play a part in maintenance of osmotic tension of the semen but its precise role has not been demonstrated. Estimations of levels of citric acid were used in studies of the effect of under feeding in bulls (Mann and Walton, 1953) in order to measure the effects of this on the accessory glands of the reproductive tract.

C. Resistance to Environmental Changes

(i) Low temperature storage. Early reports dealing with undiluted semen, reviewed by Anderson (1945), indicated that the survival rate of undiluted semen, cooled slowly to 5°C., could not be closely correlated

with initial motility and there was no clear evidence that this alone could be used as a guide to potential fertility. Although Swenson and Herman (1944) had demonstrated a highly significant linear correlation (r = 0.84) between survival of undiluted semen at 5°C. and fertility, Madden et al. (1947), with limited insemination results in an experimental station herd, were unable to confirm these findings.

Using diluted semen, Buckner et al. (1954) found only a low correlation between spermatozoal survival at 4°C. and fertility in the 72 samples studied, but between bulls this correlation was higher than that within bulls. These authors recommended that motility estimation need only be performed up to the 4th day of storage; subsequent estimations were found to be of no value. The difficulty in deciding on the end point of the test could thus be eliminated. Erb et al. (1950) and Weeth and Herman (1949) had previously reported similar findings. Also recently Bratton, Foote, Henderson, Musgrave, Dunbar, Dunn and Beardsley (1956,a) using semen diluted at 1:100 and 1:300 confirmed this correlation to be of little practical value.

(ii) High temperature viability tests. Ludwick, Olds and Carpenter (1948) reported a high correlation between time of incubation at 100°F. required for all spermatozoa to lose their motility and conception rate. Similar results, on a between bull basis, were also reported by Buckner et al. (1954) when they estimated the progressive motility after incubation for 16 and 28 hours in yolk-citrate at 38°C. in their research laboratory but, on repeating these observations at an insemination centre, they obtained a lowered correlation coefficient.

As in all survival tests, the estimations are subjective and not strictly comparable. Also, where duration of survival is recorded, the end point is not always easily defined; this applies when the end point is taken as the complete cessation of motility or as the absence of progressive motility.

(iii) Resistance to temperature shock. The effect of cooling on diluted and on undiluted semen was reviewed by Anderson (1945). Lasley and Bogart (1943), after observing the spermatozoal survival rate with diluted and undiluted semen cooled suddenly from 30°C. to 5°C., claimed there was a relationship between the survival rates in the diluted semen, subjected to cold shock, and fertility. This experiment was repeated by Bishop et al. (1954), who, using the nigrosin/eosin stain solution at different temperatures, were able to assess the effects of the cold shock by counting the numbers of stained spermatozoa. They showed that the initial motility and fertility of the samples tested increased with increasing resistance to temperature shock, and instanced this finding as evidence of a qualitative difference in the living spermatozoa. However, they pointed out that the use of a hypotonic stain solution could also have influenced their findings (see page 59). Only a multiple correlation on a between bull basis between resistance to thermal shock, spermatozoal density and fertility was reported by Stone et al. (1950).

(iv) Resistance to sodium chloride and dilution effect. The measurement of the deleterious effect of a 1 per cent solution of sodium chloride on spermatozoal survival, as reported by Milovanov (1934), was described in detail by Anderson (1945). Subsequently,

Cheng et al. (1949) and Emmens and Swyer (1948), using rabbit spermatozoa, showed that a similar effect could be obtained with chloride-free diluents. No extensive studies of this phenomenon for semen quality assessment appear to have been made, although it would seem to be worthy of further investigation in view of variations in isotonicity of the different diluents that have been tried for semen storage.

D. Physical and Other Methods of Assessing Semen Quality

(i) Impedence change frequency (I.C.F.). This objective method of assessing spermatozoal activity, which was devised by Rothschild (1948), depends on the frequency of changes in resistance to the passage of an electric current through the sample of semen. However, these measurements, which were later shown by Rothschild (1950,a) to be significantly related to visual motility estimates, are only reliable in semen with a wave motion and cannot be applied to diluted semen. Cummings (1954), in an extensive investigation into the relationship between impedence change frequency and conception rates with over 35,000 first inseminations, found it to be of value in assessing semen quality. Bishop et al. (1954) also reported a direct relationship (significant at the 5 per cent level of probability) between I.C.F. and fertility in the overall results of their survey. There is no evidence that the semen is damaged by this test, which could therefore be done on the actual semen to be used in the field.

(ii) Objective measurements of motility. A photographic method of recording the movement of spermatozoa was reported by Rothschild (1953). This method, which is expensive and time consuming, shows up only the

moving spermatozoa but it does enable their rate of travel to be measured. Moeller and Van Demark (1955), using a stop watch, were able to estimate the speed of selected cells passing across a microscopic field, but the spermatozoa selected for this measurement may not have been representative of the semen samples studied. A further modification to obviate this possible discrepancy was reported by Baker, Cragle, Salisbury and Van Demark (1957), who measured the time required for a given number of cells to pass over a ruled line in a Petroff-Hausser chamber on a microscope stage; subsequently, the number of non-motile spermatozoa in the chamber were also counted. Possible errors, as pointed out by the authors, could arise from the dilution effect on the spermatozoa, which could only be counted at a concentration of 25,000 per mm^3, and from the velocities obtained by this method since these were based on the assumption that the spermatozoa travelled in straight lines.

Bosselaar and Spronk (1952) devised a photoelectric apparatus that automatically counted the number of spermatozoa passing over a small aperture in a given time, and that also measured photoelectrically the total number of spermatozoa on the slide. However, certain difficulties were subsequently reported (Bosselaar, Spronk and Van Dam, 1955) with the apparatus and with the sampling procedure. Rikmenspoel and Van Herpen (1957) continued this work by using an apparatus designed to make photographic, as well as photoelectric, measurements so that normal and abnormal types of spermatozoal movements, as well

as measurements of the speed and numbers of moving cells could be detected. In spermatozoa with no morphological defects a type of movement classed as abnormal was recorded, and the numbers of motile spermatozoa were obtained with a high degree of accuracy. No fertility studies were reported with semen tested by this apparatus.

Although the above objective methods show some promise, they have not been extensively investigated. Furthermore, the results already obtained have not been correlated with fertility studies. There is also some doubt as to whether they have, in fact, any practical value for use at an insemination centre; the cost of the equipment and time required to do the tests are both considerable.

(iii) Other objective semen studies. Lindahl (1956) reported an attempt to separate mature and over-mature spermatozoa by a counter-streaming centrifuge. Studies on the rate of heat production by spermatozoa were referred to by Bertaud and Probine (1956) and by Clarke and Rothschild (1957). Uterine mucus from the cow was claimed by Binello (1954) to increase spermatozoal survival, but in 230 samples tested there was no relationship between viability and fertility. Studies on electrophoresis of 50 samples of bovine semen, as described by Vesselinovitch (1959,a), did not reveal any differences in the seminal plasma of sterile and fertile bulls, but the amounts of the various components varied, not only between different animals, but also between different samples from the same animal. Pernot and Szumowski (1958) claimed that the presence of large amounts of albumen detectable by electrophoresis could indicate impaired spermatogenesis.

E. Combined use of several tests for semen quality assessment.

Various tests have been studied by several workers in order to assess their value, used either singly or in combination, as indicators of the potential fertility on a between bull or between semen sample basis. Bishop et al. (1954) obtained the most significant correlations in their overall data between conception rate and (a) the incidence of dead spermatozoa, as measured by the nigrosin/eosin staining technique, and (b) impedence change frequency, and concluded that, in their study, the physical activity of the semen was more closely related than its metabolic activity to fertility. These authors were of the opinion that, even with refinements in techniques to improve their accuracy, the more complicated metabolic measurements would be unlikely to be of any more value than the above physical measurements. These findings were, in the main, confirmed by Cummings (1954), who used 508 samples (from 56 bulls) to study the correlation between fertility and the following charactors: impedence change frequency, motility rating, and live/dead counts. Buckner et al. (1954) confined their series of investigations to simple tests that could easily be adopted for routine use at an insemination centre. They obtained only low correlations with fertility, and indicated that these promised to be of more value in between bull rather than within bull studies. The particular tests of value were motility after incubation at 37°C. and a combination of the results from methylene blue reduction, motility drop after storage in 3 per cent anilene blue solution at 4°C. and initial motility at 4°C. in yolk-citrate

diluent. For routine insemination work Bratten et al. (1956) recommended the estimation of the total spermatozoal concentration and of the percentage of motile spermatozoa, the latter being assessed microscopically in a low dilution prepared soon after collection. The probable numbers of motile spermatozoa in the final dilution were calculated from these measurements, which these authors claimed could be used to select ejaculates on the basis of the predicted fertility for the different levels of dilution and numbers of living spermatozoa. However, the reliability of these estimates under routine conditions has not been demonstrated. Erb, Flerchinger, Ehlers and Mikota (1955,a) concluded that the various metabolic tests were of some value but more experimental work was required to establish the exact relationships between spermatozoal concentration and motility and the metabolic test results. They also emphasized the need for standardisation of the methods.

F. General remarks on semen quality assessment. The findings in this work to date suggest that among bull rather than within bull correlations between semen tests and fertility are more likely to be obtained. With metabolic tests the procedures must be carefully standardised, but there is always the possibility that interactions with other factors not being recorded may influence the results. These detailed tests are not readily applicable for use at an insemination centre. Motility tests have been found to show some correlation with fertility, but these are in the main subjective and the results obtained by different workers are therefore not comparable; further investigation

of the objective measurements of the character of motility could therefore be of value. With the use of the nigrosin/eosin differential stain, the technique must be carefully standardised and it would appear preferable for the actual counts to be done by one experienced operator. Also, such a technique differentiates live from dead spermatozoa and not necessarily motile from non-motile spermatozoa. A sample containing only a low percentage of actively motile spermatozoa could possibly have a higher potential fertility than one containing a higher percentage of living but non-motile spermatozoa. Finally, the laboratory test cannot measure fertility differences occurring either through the handling, dilution and insemination procedures or, what is probably most important, through variations in the fertility due to disease in the cows or in the bulls or to the management of the cows inseminated. Strict attention to semen handling and insemination techniques will reduce their influence on conception rates to a minimum and lead to an increase in the proportion of conception rate variations detectable by laboratory tests. At present it is doubtful if more than 20 per cent of the differences in fertility can be accounted for by variations detectable in the laboratory. Since the fertility of bulls at an artificial insemination centre tends to fall within a narrow range (e.g. 10 per cent), the difference detectable by these tests cannot be expected to be great.

G. Sex determination. Lindahl (1958) indicated that, since the spermatozoa carrying the X and Y chromosomes were of different weights

they could be separated by counter-streaming centrifugation at different speeds; when semen was centrifuged thus at 1000 to 1100 r.p.m. and the 'heavy' spermatozoa, retained in the separation chamber, used for the insemination of 142 cows 42.9 per cent of the calves were males; however, when the 'light' spermatozoa, leaving the separation chamber of the centrifuge running at 1100 to 1200 r.p.m., were inseminated into 121 cows 58.7 per cent of the calves were males. These differences in the sex ratio were not significant and repetition of this on a wider scale is awaited. The possible effect of the pH value of the blood of the male on the sex ratio, as suggested by Mc.Whirter (1956), has not been so closely studied. It has also been claimed that the sex ratio could be effected by a lowered general level of fertility (Guse, 1957), or by an acid pH, as got in stored semen (Schwarz, 1955). However, in a study of 17,332 calvings Baier and Haeger (1958) found no indication that either stored semen or the individual bull had any effect on the ratio, which was 123:110 (males:females). Studies on the separation of the male and female producing spermatozoa of the rabbit by electrophoresis have been described by Schroder (1941) and more recently by Gordon (1957). It was also shown by Macpherson and Vesselinovitch (1959), in a study with 4 bulls, that semen in a milk diluent could be electrophoretically treated without any effect on its fertility.

Although Vesselinovitch (1959,b) observed that bovine spermatozoa could be separated into two fractions in an electrophoretic cell, he claimed that this separation was not as straightforward as suggested

by some previous authors. He reported that, whereas immotile spermatozoa migrated in one direction only, motile spermatozoa reacted in several different ways, depending on their degree of motility and on the intensity of the current applied. No fertility studies were reported, but these results gave a possible explanation for the previous conflicting reports of other workers.

SECTION V

FACTORS AFFECTING SPERMATOZOAL SURVIVAL

Ejaculated semen has as its two main components, spermatozoa, which are produced in the testis and stored in the epididymis, and seminal plasma, which is produced from the accessory glands and mixed with the spermatozoa only at the time of ejaculation. The production of semen is controlled by the testicular and pituitary hormones and variations in its composition, even within the individual animal, are only to be expected since so many glands in the genital tract play a part in its formation. The structure of spermatozoa has been described in detail by Mann (1954). The head of the spermatozoon is composed mainly of deoxyribonucleic acid and its anterior part is covered by the acrosome cap. Running through the mid-piece and tail of the spermatozoon, and surrounded by a spiral sheath, is the axial filament, which, being the main contractile element, is responsible for its motility. The enzymes, responsible for the aerobic and anaerobic metabolism of the spermatozoon, and therefore for its motility and respiration, are located in the mid-piece region. The constituents of, and the metabolic changes in, semen have been studied extensively in view of their possible importance in semen dilution and preservation and these will now be considered briefly.

A. The effect of certain compounds found in semen on fertility and on spermatozoal survival and their value when added to diluents

(i) Hyaluronidase, other proteins and amino acids. The enzyme

hyaluronidase appears to be associated with the head of the spermatozoon. There have been many studies made of the effect of this enzyme in laboratory animals but its precise role has not been determined. Austin (1948) suggested that it might be concerned with the penetration of the spermatozoon into the egg while Yamane (1956) thought that it might be important in altering the viscosity of the cytoplasm inside the egg itself. It was reported by Rowlands (1944) that the addition of hyaluronidase to rabbit semen improved its fertility, but Chang (1947) was unable to confirm this.

Mann (1954) pointed out that the extra-cellular protein content of the seminal plasma is rapidly acted on by various enzymes soon after ejaculation, and this must be considered in the interpretation of such an analysis. Larson and Salisbury (1954) reported that 90% of the total nitrogen was present as non-dialysable protein, largely lipo-protein or glyco-protein in nature, but further evidence on the stability of these was required. Although proteins are contained in the commonly used diluents, there is a need for more information on the functions of the proteins normally present in the seminal plasma. Gassner and Hopwood (1952) demonstrated the presence of the amino-acids, serine, glycine, alanine, aspartic acid and glutamic acid in seminal plasma.

A protective action of seminal amino-acids and proteins on sea urchin spermatozoa was demonstrated by Tyler and Rothschild (1951), motility and fertility being retained for longer periods, but there was no increase in oxygen uptake and glycine was not utilised as a substrate. One possible explanation was that glycine may combine with the trace elements, such as copper and zinc, and so prolong vitality but such an

effect has not been demonstrated in bull semen. A protein spermatozoal anti-agglutinin, which prevents agglutination of the bovine spermatozoal heads, has been reported by Lindahl and Kihlström (1954, a,b).

(ii) Hormones. The effect of hormones on in-vitro metabolism was studied by Gassner and Hopwood (1955); testosterone, oestradiol and thyroxine were found to have varying effects on fructolysis and on respiration at different concentrations. Although the presence of oestrogens in bull semen was confirmed by Schaffenburg and McCullagh (1954), the role of these was not established. Traces of androgens were also reported to be present (Mann, 1954).

(iii) Nitrogenous bases. Although such substances as ergothionine in the semen of the boar, and choline and spermine in human semen, have been found to be of importance, these substances have not been shown to be of any significance in the bull. Recent research has shown the presence of glycerylphosphorylcholine, which is formed in the epididymis and which could act as a pre-cursor of free choline (Diament, Kahane and Levy, 1952; Lundquist, 1953; Dawson, Mann and White, 1957). The possible role of this compound in semen metabolism has not been evaluated.

(iv) Plasmalogen and fatty acids. Boguth (1952) found that bull semen contained plasmalogen in a concentration of between 0.3 and 0.9 mg. per ml., two thirds of this being within the spermatozoa. Hartree and Mann (1958) discovered that the lipid component of ram spermatozoa was mostly plasmalogen, which was the source of fatty acid and not lecithin as previously thought. Acetic and formic acids were identified by Flipse and Potter (1955) as the main fatty acids in seminal plasma.

(v) Deoxyribonucleic acid. Although the deoxyribonucleic acid content of the spermatozoon and other cells is constant within a species (Vendrely, 1952), there are indications that, in humans at least, it is lowered in cases of infertility and subfertility (Weir and Leuchtenberger, 1957).

Mann (1951) has shown that ribenucleic acid is not present in mature spermatozoa.

(vi) Inorganic constituents. Of the alkali metals, Lardy and Phillips (1943) showed that potassium (at least 0.005 M.) and magnesium (0.012 M.) without calcium were necessary for motility. White (1953,a,c) reported that the addition of 0.004 M. potassium chloride to the diluent improved the glycolysis and the motility of spermatozoa that had been subjected to repeated washing or dilution, and then incubated for 3 hours at 37°C.; apparently in both these processes part of the damage is caused by the loss of potassium from the cell (see page 98) but, at very high dilutions, it would appear that other substances must also be responsible for the adverse effect. White (1953,b) claimed also that high concentrations of potassium could have an adverse effect on motility. From these observations it will be appreciated that an adverse effect on motility would be unlikely to occur through a loss of potassium in semen used at low dilution rates (i.e. with spermatozoal concentration of 200 x 10^6 per ml.). At higher dilution rates (i.e. with 20 x 10^6 spermatozoa per ml.), the inclusion of 0.004 M. of potassium in the diluent improved the motility, but at very high dilution rates (i.e. 2 x 10^6 spermatozoa per ml.) no beneficial effect of this addition was demonstrated. This addition of potassium was not studied in semen stored at refrigerator temperatures neither were fertility trials carried out.

The reports of the effect of calcium have been somewhat conflicting. Lardy and Phillips (1943) and Blackshaw (1953,b) claimed that Ca ions depressed motility, but this was not in accord with the work of Bishop (White, 1958). The need for magnesium in semen diluents has not been clearly shown, but the possible effects of iron, copper, lead and other heavy metals on spermatozoal motility were studied by White (1955,b). He concluded that, whilst iron and copper appeared to be the most toxic, it was unlikely that these would be present in sufficient quantities in normal water supplies to have any effect. The hitherto generally accepted recommendation that only distilled water should be used for diluent preparation would not, in the light of this report, appear to be well founded, but the effect on fertility would need to be investigated. Chelating agents were used by White (1955,a) to induce trace element deficiency in spermatozoal suspensions in order to investigate the need for such elements. However, certain of these chelating agents were found to be toxic to spermatozoa. Van Koetsveld and Spruyt (1959) found no correlation between fertility and the manganese or copper content of semen.

(vii) Vitamins. Although ascorbic acid has been found to be present in levels as high as 14 mg./100 ml., its function is not clear. Phillips, Lardy, Heiser and Ruppel (1940) claimed that the fertility of the bull varied with the level of seminal ascorbic acid, but Jacquet, Cassou, Plessis and Brière (1950) found no relationship between the ascorbic acid content and the quality or fertility of semen. However, Mann (1954) could not accept the assumption of these workers that the reducing power of semen was, in the main, attributable to ascorbic acid, and the work of

Larson and Salisbury (1953) also opposed this theory. No beneficial effect on fertility of the addition of ascorbic acid to semen has been reported.

The presence of riboflavin in washed bull spermatosoa was reported by Lardy and Phillips (1941) and by Van Demark and Salisbury (1944); the yellow colour of semen from certain bulls is of composite origin, but it is probably due to flavins from the seminal plasma and not from the spermatozoa (Mann, 1954). Van Demark and Salisbury (1944) also reported the presence of traces of other vitamins of the B complex, and White (1954,a) has claimed that biotin improved spermatozoal survival when added to a fructose phosphate diluent.

(viii) Adenosine triphosphate. Spermatozoa were shown to cause a breakdown of exogenous fructose to lactic acid both aerobically and anaerobically by the usual phosphorylated pathway (Mann, 1945, a,b). The breakdown of adenosine triphosphate (ATP) to adenosine diphosphate (A.D.P.) is considered to supply the energy for the contraction of the spermatozoal tail fibrils, which are responsible for the motility of the spermatozoa, and the ATP utilised in providing the energy for movement is replaced during fructolysis. A close correlation between the ATP content of semen and its motility was demonstrated by Mann (1945,a,b).

It was realised as long ago at 1940 (Chang and Walton, 1940) that low temperature had an adverse effect on spermatozoa and this effect was termed "cold shock". Mann and Lutwak-Mann (1955) found that the low temperature caused a loss of the adenosine triphosphate and also a leakage of certain proteins from the spermatozoa. The acrosome cap is also known to become detached when the spermatozoa are cooled (Hancock, 1952).

B. The effect of the metabolic and physical state of the semen on spermatozoal survival

(i) Methods of inactivation. Although the lowering of the temperature has been the basis of semen preservation, investigations have been reported on the use of other physical or chemical methods either alone or in combination with low temperature storage.

Semen metabolism can be reduced to a low level by conventional storage methods but there is still a need to provide exogenous substrates in the diluent, and the latter must be able to deal with changes in the medium arising from the end products of the cell metabolism.

The inhibition of spermatozoal motility by storage in a yolk glucose lactic acid diluent at pH 6.45 and its reactivation by sodium hydroxide or sodium bicarbonate was reported by Willett and Ohms (1958,b), but their results were inconclusive. Howe and Flipse (1959) used fluoracetate, in concentrations up to 0.01 M., to prolong the life of spermatozoa, but this did not effectively inhibit motility and glycolysis. Norman, Bortoff and Dunbar (1956) had previously used iodoacetate for this purpose, but the loss of motility observed with this additive may have been due to the toxic effect of the H_2O_2 produced. The toxic effect of H_2O_2 on spermatozoa has also been recently referred to by Wales, White and Lamond (1959). Certain drugs of the local anaesthetic type (desexyephedrine and tutocaine) were reported by Asher and Kaemmerer (1950) to stimulate spermatozoal motility, but certain allied compounds are also toxic to spermatozoa.

(ii) The effect of variations in the oxygen tension. The in vitro

studies by Walton and Dott (1956) have shown that optimum spermatozoal motility occurs under aerobic conditions which appears, according to the work of both Campbell (1932) and Bishop (1956) who worked with rabbits, to be the condition in-utero. Furthermore, the perfusion experiments described by Walton and Dott (1956) confirmed that, although spermatozoal metabolism could be reduced by anaerobic storage, a supply of utilisable carbohydrate and a means of buffering the acidic end products of its metabolism must be provided. Salisbury and Sharma (1957) studied the correlation between semen metabolism and routine A.I. storage practices, which involve partially anaerobic conditions using 0.9 per cent sodium chloride and phosphate diluents. Their results confirmed that the diluent must provide utilisable substrates, be able to eliminate the toxic products of the metabolic changes and also exert a protective action against cold shock and dilution. It must be pointed out, however, that the above studies were made at 37°C., while the effects could safely be assumed to be less at the normal storage temperature of 5°C., no studies at the latter temperature have been recorded.

Henle and Zittle (1942) and Lardy, Hansen and Phillips (1945) showed that the metabolic characteristics of epididymal spermatozoa, although varying between samples, were markedly different from those of ejaculated spermatozoa; the former showed a lower rate of endogenous respiration and a higher level of oxidative metabolic efficiency than the latter. Later, Lardy Ghosh and Plaut (1949) reported that this was due to the presence of a metabolic regulator, which was inactive

in the epididymal spermatozoa and only became activated after the spermatozoa had been ejaculated and, therefore, mixed with seminal plasma. Although Ghosh and Lardy (1952) isolated from boars' testes a yeast stimulating factor which they claimed to be elemental sulphur, it had no stimulating effect on epididymal spermatozoa; however, respiration and aerobic glycolysis were both enhanced following the addition of hydrogen sulphite or sodium sulphite. Since a number of sulphydryl compounds (e.g. cysteine) are known to increase the respiration and glycolysis of epididymal spermatozoa, (Rodel, 1955), it is possible that the effect is due to one of these compounds being released at ejaculation. There is a need for further research into this stimulating mechanism, ~~and~~ into the conditions under which the cells are stored in the epididymis and into the maturation of the spermatozoa.

(iii) Osmotic pressure and electrolytes:- Milovanov (Anderson, 1945) suggested that ~~although~~ sodium chloride caused over- activity of the spermatozoa; it also damaged the lipid capsule and led to early death of the spermatozoa. In view of this observation, a reduced electrolyte content and an acid media were said by Anderson (1945) to favour spermatozoal survival.

However, Blackshaw and Emmens (1951) showed that, provided the diluents were buffered and isotonic, motility was not affected by changes in the levels of glucose and sodium chloride in the diluent. Although these authors showed that spermatozoal survival was greatest in isotonic diluents, it was less affected at all pH levels by hyper- than by hypotonicity, but the effect also depended on the

dilution rate of the original semen samples. This work was not confirmed by Brochart (1951) who, under anaerobic conditions, obtained the best survival after dilution with up to 60 per cent distilled water and who claimed this ensured that the osmotic pressure within the cell remained normal, storage being at 4°C. Salisbury and Nakabayashi (1957) reported an increased oxygen uptake and motility with reduced fructolysis and lactic acid production in the presence of a sodium chloride diluent. A protective action of the sulphate ion against the deleterious effect of normal saline on the spermatozoal capsule was claimed by Milovanov (Mann, 1954); this has not been extensively used in diluents but Emmens and Swyer (1948) could not confirm this with rabbit spermatozoa.

With semen stored at 37°C. Bishop and Salisbury (1955,a) found that a saline phosphate diluent adversely affected spermatozoal respiration and motility, whereas with a yolk-phosphate saline diluent only motility was depressed (Bishop and Salisbury, 1955,b). Rothschild and Barnes (1954) in a quantitative study of the constituents of the seminal plasma of 10 bulls found that its freezing point depression was -0.533, and, therefore, many of the diluents in routine use were not isotonic with the semen. No differences were observed between breeds or between bulls of different ages. Similar findings were reported from the National Institute for Research in Dairying, Report (1953,b) in which an osmotic equilibrium between semen and blood of bulls was also demonstrated.

Although the use of a non-electrolyte, e.g. glucose, to maintain

isotonicity when reducing the chloride content of the medium has been shown to improve motility and survival (Blackshaw and Emmens, 1951; Kampschmidt, Mayer and Herman, 1953,b), this has not been investigated extensively in fertility studies.

(iv) pH value. Salisbury and Kinney (1957), with semen diluted in a phosphate buffer containing Na, K, Mg and chloride ions found that the higherthe pH value, the higher was the lactic acid production, but part of the variation in pH value at least was associated with individual ejaculates. Norman, Johnson, Porterfield and Dunbar (1958,b) used a coconut-milk diluent with either sodium citrate or calcium carbonate and storage at room temperatures. These authors claimed that at the pH value of these diluents (5.5 to 5.8) spermatozoal metabolism was maximally inhibited, but the effect could be reversed by the addition of alkaline coconut milk diluent, even after several days of room temperature storage at this low pH. With semen diluted in the Illini Variable Temperature (I.V.T.) diluent (see page 113) and stored at 37°C., (Salisbury and Van Demark, 1957) showed that CO_2 could act as a reversible inhibitor of glycolysis, this depressing effect on metabolism being reversed by gassing with 95 per cent nitrogen and 5 per cent CO_2. In a subsequent publication, Cragle and Salisbury (1959) confirmed this and demonstrated that at 37°C., with the osmotic pressure and level of cations (i.e. Na, K and Ca) constant and pH value of 5, as opposed to one of 7.5, there was initially a higher rate of fructolysis than that observed at pH value of 7.5, but, on continued exposure, fructolysis ceased at the lower

pH value but continued unchecked at the higher pH value. Using a flow dialysis technique at room temperature, Sharma (1957) found that the I.V.T. diluent gave best survival. Similar findings on the inhibitory effect on metabolism, following storage at low temperature in a CO_2 atmosphere, were reported by Senegacnik (1958).

In an investigation into the metabolic activity of spermatozoa at different temperatures, Blackshaw, Salisbury and Van Demark (1957,b) found that the fructose uptake decreased more between 37°C. and 21°C. than between 21°C. and 5°C. However, O_2 uptake dropped more rapidly at the lower temperatures. At 37°C. in phosphate diluent, fructose utilisation and lactic acid production were higher than in sodium chloride, but O_2 uptake was higher in the latter. However, these metabolic differences were not found at 21°C. or at 5°C., indicating the need for study of these metabolic processes at different temperatures in diluent investigations. Although it is generally accepted that a pH value of approximately 7.0 gives the optimum survival rates, Phillips and Lardy (1940) advocated a pH of 6.75 for the buffered yolk-phosphate diluent. Kok (1953,a), using a citrate-fructose diluent without egg yolk, obtained the best survival rates at pH 6.75 and 6.25, the former being satisfactory for storage up to 24 hours, and the latter being required for longer storage periods. Johnson, Flipse and Almquist (1956), however, using a skim milk powder diluent, found a highly significant interaction between pH value and electrolyte content, as judged by the effect on spermatozoal motility; such a study has not been reported with other diluents, ~~however.~~ The semen was diluted and cooled to 4°C. and, over the 12

days storage period, no changes in pH value or osmotic pressure of the diluent were recorded.

Although the pH value and electrolyte content need not be kept at a certain critical level for maximum survival, it would appear that their effect is influenced by the dilution rate and also the quality of the semen. However, these changes were not studied in fertility trials.

Considerable variation in the pH value of semen at collection has been reported in the literature, but there is no evidence of any effect on fertility within the range of 6.5 to 7.5. Moreover, this range is understandable since the amount of secretion from the different accessory glands will vary in the different samples and this is not obviously of any great importance in natural mating.

The buffering capacity of semen was reviewed by Anderson (1945) (1946), who showed that whole semen was more completely buffered on the acid than on the alkaline side of neutrality and that pH change in whole semen was related to duration of motility.

C. The Effect of Dilution on Spermatozoa

The studies of the effects of dilution of the semen of the lower forms of animal life were reviewed by Mann (1954) but the changes involved, commonly referred to as the "dilution effect", are not yet fully understood. In particular, attention has been paid to the work of Schlenk and Kahmann (1938) who claimed that the increase in motility of trout spermatozoa, after dilution with water or with a sodium chloride solution but not with a potassium chloride

solution, could be due to the transfer of the intra-cellular potassium to the surrounding medium. The possibility of a similar mechanism being responsible for the activation of bull spermatozoa has also been studied. A high potassium concentration of the medium surrounding the cells in the epididymis was reported by Sørensen and Andersen (1956). By carrying out sodium and potassium estimations on the spermatozoal cells and plasma in the testis and in the subsequent parts of the tract up to the point of ejaculation, Salisbury and Cragle (1956) found that the sodium: potassium ratio showed a shift from 1:2 in the efferent ducts in the testis to 1:1 in the ampulla, this being due to a marked increase in the sodium content in the epididymis; the plasma in the efferent duct of the testis was hypertonic but this was apparently decreased in the epididymis until it was finally isotonic at ejaculation. These authors also postulated that this change allowed water to enter the cell due to its increased permeability, which in turn initiated motility. This work indicated that a transfer of the potassium ion may play a part in the initiation of motility in the bovine semen and also a need for further studies on the inclusion of potassium in diluents. The pattern of the dilution effect appears to be an initial but variable period of activation, followed by a gradual loss of motility and exhaustion of the cells. This dilution effect has been found to vary in different diluents. Rao and Hart (1948) reported that in normal saline at a dilution of 1:400 bull spermatozoa showed an initial stimulation but this was followed by a loss of motility, most of the spermatozoa being

immotile after 2 hours; no such adverse effect on motility occurred in the yolk-diluent at the same high dilution rate. Blackshaw (1953a), using dilute semen suspensions, found a highly significant drop in motility with increasing dilution rate in a sodium chloride-glucose diluent. Also in ram semen the biochemical changes, brought about by repeated washing of the raw spermatozoa, have been found to be somewhat similar to those due to the dilution effect (Mann, 1945,a). According to White (1953,a) the metabolic effect of dilution is confined to glycolysis, there being no effect on respiration. Also Ehlers and Erb (1956) found that the glycolysis of semen was influenced by dilution rate, but Salisbury (1946,a) showed that oxygen was more harmful to low than to high concentrations of spermatozoa. Cheng, Casida and Barrett (1949) demonstrated that the effect of dilution could not be reversed by centrifuging and reconcentration of diluted samples; the fact that this did not restore the original motility of the undiluted semen may be taken to indicate the possible diffusion of some essential element out of the cells. These authors also showed that this effect was not due to a mere oxygenation of the spermatozoa. Salisbury, Beck, Cupps and Elliott (1943,a) reported that spermatozoal survival was reduced in higher dilution rates, and also Kok (1952), with in vitro studies, found that the duration of motility was reduced with increasing dilution rates and decreasing spermatozoal concentrations; motility was also reduced in a yolk citrate fructose diluent, isotonic with bull blood and with a pH of 7.25, when compared with that in a similar diluent with

a pH value of 6.25, and with a hypertonic diluent. However, investigations reported by Schmidt and Jenichen (1954) indicated that with boiled milk as a diluent, spermatozoal motility was not appreciably affected by increased dilution rates.

D. The Effect of Female Genital Tract Fluid

Olds and Van Demark (1957) reported that, under anaerobic conditions at 37°C., semen remained motile after 9 hours in vaginal mucus, 7 hours in uterine fluid and 19 hours in follicular fluid; the survival was in general longer in mucus and uterine fluids at or near oestrus; the highest O_2 uptake was obtained in follicular fluid, but agglutination was also noticeable in this. A recent publication by Yates and Olds (1959) confirmed the stimulatory effect on respiration of the follicular fluid, which showed a marked pH change (7.4 to 8.9) during incubation due to diffusion out of its CO_2 content. An extensive study of the variation in these effects between cows would appear to be necessary in order to assess the importance of these findings.

S E C T I O N VI

DILUTION AND STORAGE MEDIA

A. Composition of and function of seminal plasma

Although the seminal plasma is the natural diluent for the spermatozoa its complete role has not been fully demonstrated. It is considered to be beneficial to the spermatozoal survival partly by its dilution and stimulatory effect (Lardy, Ghosh and Plant, 1949; Salisbury and Cragle, 1956), and partly by the possible action of specific substances contained in it, as demonstrated in the rabbit by Chang (1947, 1949). Certain aspects of these were reviewed by Anderson (1945), but further attention has been paid to the composition of seminal plasma in view of its possible importance in semen storage.

The seminal plasma is normally an isotonic and almost neutral fluid containing, amongst other substances, sodium, potassium, magnesium, calcium, chloride and phosphate ions. Larson and Salisbury (1953) showed that sulphite was also present, but its exact role was not demonstrated. Although this is the medium in which the spermatozoa are transferred to the female reproductive tract, it is not the ideal medium for spermatozoal survival, as evidenced by the early death of spermatozoa stored in it in vitro. Furthermore, since good fertility is obtained even with highly diluted semen (Salisbury, Elliott and Van Demark, 1945,a), it would appear that if seminal plasma is necessary for fertilisation at all, only traces of it are required. The work of Chang (1949) with the rabbit indicated that it was of value. Milovanov

(1934) concluded that the chemical composition of the seminal plasma should not be used as a basis for diluent preparations. However, Chang (1957) showed that in the rabbit the seminal plasma could reverse the effects of "capacitation", which, as demonstrated by Chang (1951), is the maturation process undergone by rabbit spermatozoa in the uterus before becoming capable of fertilisation. Austin and Bishop (1958) have indicated that the loosening or detachment of the acrosome cap is at least one of the major changes occurring at capacitation, but this has not been demonstrated in the bovine. Asdell (1958) has also posed the question as to whether or not the seminal plasma from a highly fertile bull could have a beneficial effect on semen from a bull of low fertility. Fractionation of the ejaculate of the bull, unlike that of the boar, is not readily possible, but Lutwak-Mann and Rowson (1955) reported on the chemical composition and possible function of the pre-spermatozoal fractions obtained by the electro-ejaculation method. They were unable to demonstrate any beneficial effect of this fraction on fructolysis or motility, and they concluded that its function was probably only to clear the urethra of cellular debris and other possible contaminants of the semen. The effect of this fraction on fertility was not investigated and it is not normally included in the samples collected for insemination purposes. However, Kinast (1958) claimed that, although the fresh pre-spermatozoa fraction temporarily increased the oxygen uptake of both ejaculated and epididymal bull spermatozoa, if this fraction was stored then it adversely effected the spermatozoa.

The addition of fresh seminal plasma to stored semen was claimed by Szumowski (1952) to improve motility, to reduce the pH and to increase the number of motile spermatozoa. In contrast to this, Flipse (1954) found that the presence of seminal plasma caused an apparent reduction in the uptake of glucose C^{14} by spermatozoa.

B. Basic requirements for a diluting fluid

The chief considerations governing the choice of a diluting fluid as outlined by Anderson (1945) could be usefully recalled at this stage. These were listed as: (a) osmotic tension, (b) pH value (c) buffering capacity, and (d) non-toxicity with a correct balance of electrolyte and non electrolytes, cations and anions. To these must be added : (i) stability of the diluent even after prolonged storage, (ii) presence of substrates to support the reduced metabolism of the stored spermatozoa, and (iii) no detrimental or inhibiting effect on antibiotics, which are now accepted as being essential in diluting fluids. Lastly, the diluent should be easily and cheaply prepared, permit the microscopic examination of the spermatozoa in a clear field and not render difficult the cleaning of the glassware, or other semen containers.

Probably the most important considerations in diluent studies are the length of time that the diluted semen will be stored prior to its use for insemination, and the actual semen dilution rates to be used. In general, conception rate studies with semen used only on the day of collection do not give much useful information. Also many of the diluent trials have not been carried out at sufficiently high dilution rates or with semen stored long enough to enable the diluent to be critically

assayed. Since semen quality, semen dilution rate, length of storage of diluted semen, efficiency of the techniques, and even methods of conception rate assessment can be reflected in the fertility results, these factors must also be considered when assessing the merits claimed for a particular diluent. The spermatozoal survival rates of diluted semen appear to bear a variable relationship to the actual fertility of the semen and the only reliable test of a dilutent is, therefore, the controlled measurement of its effect on conception rate.

Although in the early development of artificial insemination the conventional egg yolk diluents gave satisfactory results after storage for up to 3 or 4 days after collection, it would appear that with the adoption of high dilution rates, although these do not usually affect the fertility of semen stored up to 30 hours, the drop in fertility of semen after storage for long periods precludes its use in commercial insemination practice. There is, therefore, a need for diluents which will extend the period over which semen can be stored at normal refrigerator temperatures.

C. Composition of the different diluents used

(i) Diluents containing egg-yolk. The widespread development of artificial insemination was largely made possible by the work of Lardy and Phillips (1939), who demonstrated the protective action of egg yolk against cold shock. The active principle in yolk, responsible for this, is now thought to be lecithin or a similar phospholipid occurring either free or in combination with protein

(Mayer and Lasley, 1945; Kampschmidt, Mayer and Herman, 1953,a; Blackshaw, 1954). Lipo-protein was also claimed by Kampschmidt et al. (1953,a) to be more effective in a citrate or phosphate than in a glucose medium, but it is of interest to note that this action of lecithin had already been reported several years previously by Milovanov and Selivanova (1932). The protective action of yolk has also been studied by Bogart and Mayer (1950), and recently Bomstein and Steberl (1959) claimed that crude lecithin had a greater protective action than purified lecithin.

The universally used diluents, which contain either egg yolk or milk as the essential constituent, have not been selected because they meet with the above requirements. The osmotic tension of some yolk diluents, giving apparently satisfactory conception rates, has, in several instances, not been found to be isotonic with semen, (Salisbury, Knodt and Bratton, 1948; Rothschild and Barnes, 1954; and Smith, Mayer and Herman, 1954). However, the pH value the buffering capacity and the electrolyte/non electrolyte balance of these conventional diluents appear in the main to be within the assumed required range. The studies reported with various diluting fluids are not always strictly comparable since they have not always been carried out under similar conditions.

Several workers have studied the composition of egg yolk, presumably with a view to developing synthetic diluents, but the methods of isolation of what was considered to be the essential fractions vary somewhat. Mayer and Lasley (1945) claimed that the acetone, alcohol and ether-

insoluble fractions had this protective action; Walton (1947) reported complete protection could be obtained with the ether-soluble fraction; and Blackshaw (1954) found that the alcohol-soluble, acetone-insoluble fraction was also protective and that it contained lecithin. Since the latter protects against cold shock in concentrations as low as 0.12 per cent (Boguth, 1952), this may possibly account for the different findings reported above.

In addition to this protective action against cold shock, egg yolk contains certain substrates that will support spermatozoal metabolism. Its glucose content can be utilised by spermatozoa (Van Tienhoven, Salisbury, Van Demark and Hansen, 1952); it has been shown by Smith, Mayer and Merilan (1956) to play a part in the dehydrogenase reactions and so maintain the spermatozoa in a reduced state. This action was found to be associated with the acetone-soluble fraction and these authors reported that the physical protective action was due to the lecithin, while the factor responsible for spermatozoal survival was associated with cholesterol. The beneficial effect of the other constituents such as vitamins, enzymes, proteins and fats has not been fully investigated. Tosic and Walton (1947) demonstrated <u>in vitro</u> that, whilst egg yolk enhanced the respiration of spermatozoa, it also contained a factor which could be metabolised by the spermatozoa to produce an inhibitor of respiration. Dialysis of the egg yolk was found to remove the source of this toxic factor, and semen used in a diluent, prepared with the non-dialysable fraction, had a conception rate equal to that used in the complete yolk diluent. Subsequently, Tosic and Walton (1950)

produced evidence that the inhibitor of respiration and motility, mentioned above, was hydrogen peroxide, which was produced in the presence of oxygen by the action of the spermatozoal enzyme system on three specific amino acids normally present in the dialysable fraction of the egg yolk. The extent to which this occurs under normal conditions of storage is not known, but hydrogen peroxide accumulation can be reduced by catalase and also by pyruvate, which is formed in spermatozoal metabolism; Van Demark, Salisbury and Bratton (1949) demonstrated that survival of spermatozoa in diluents in vitro could be improved by the exclusion of oxygen from the storage tubes and also by the addition of catalase to the diluted semen; it was postulated that this was a result of the prevention of formation of hydrogen peroxide. In contrast with the foregoing, Romjin (1950), found that the oxygen uptake of spermatozoa was maintained at a higher level in the presence than in the absence of the yolk. Van Demark, Bratton and Foote (1950) were unable to demonstrate any improvement in conception rate with the addition of catalase to the normal yolk citrate diluent, but it is possible that, under their conditions of storage, toxic levels of hydrogen peroxide might not have accumulated even in the absence of catalase.

Apparently, therefore, it is the physical protective action of the non-dialysable part of the egg yolk that plays the major role in spermatozoal storage, and any spermicidal effect, due to the breakdown of certain amino acids in the presence of oxygen, is greatly overshadowed by the above beneficial effect.

Although there is a possibility of variation in the composition of the egg adversely affecting the diluent by, for example, causing agglutination of the spermatozoa (Kok, 1953,c), such an occurrence would not be readily detected and this may account for the absence of much evidence of this in the literature. Most centres prefer to use fresh eggs wherever possible, but there are few data on the possible effect of age of the eggs. Meglioli, Pozzi, and Olgiati (1955) found no obvious effect on the motility of 52 semen samples in a diluent containing yolk from eggs up to 20-25 days old, and Morozov (1951) claimed that eggs even 1 to 2 months old did not affect spermatozoal motility provided that the yolk was of uniform colour, and that it could be easily separated from the white. In this connection, Maurice and Fidanza (1954) showed that, although there is a difference in osmotic pressure between the yolk and white, the outer layer of yolk cells appeared to prevent diffusion from the white into the mass of the yolk cells even after storage of the eggs for 200 hrs. at 1°C. The use of a chick embryo diluent has been reported by Frank, Smith and Eichhorn (1941), by Salisbury, Zelaya and Van Demark (1945b) and by DeSilva (1954) and, although <u>in vitro</u> studies have shown some beneficial effects with some samples, it may be concluded that, in view of the difficulty in preparing it, the possible benefit was insufficient to warrant its use at commercial insemination centres. Basu and Berry (1948) reported that turkey egg yolk was as effective as hen egg yolk in the storage of spermatozoa.

An interesting observation that the agglutination of spermatozoa

could occur in an egg yolk-citrate diluent, the yolk of which was of a pale yellow colour indicating a carotene deficiency in the hen's diet, was reported by Jahnel (1954). This agglutination occurred more readily with diluents of a low pH value and it varied with different ejaculates from the same bull. Heating the diluent at 60°C. for 30 minutes was said to prevent this occurrence. This finding was in accord with the report of Kok (1953,b), who attributed agglutination also to a decrease in the concentration of seminal plasma. It was more likely to occur at high dilution rates and could be prevented by the addition of seminal plasma; it was indicated that a 1:5 dilution rate should not be exceeded but this is not readily practical.

(ii) Phosphate-yolk diluent. The original yolk diluent, described by Lardy and Phillips (1939), consisted of equal volumes of egg yolk and a phosphate buffer (2.0 g. Na_2HPO_4 12 H_2O, 0.2 g. KH_2PO_4 in 100 ml. diltilled water) and its field use was reported by Willett, Fuller and Salisbury (1940). This diluent had one serious disadvantage in that owing to the presence of large fat globules it was impossible to observe the motility of the individual spermatozoa under the microscope but, nevertheless, the phosphate diluent by its depressing action on spermatozoal metabolism, and by its high buffering capacity, which maintains the required pH value during storage, is suitable for semen preservation under aerobic conditions

(Bishop and Salisbury, 1955,a). A modified yolk-phosphate buffer was also used in metabolism studies by Romijn (1947). As it has now been largely replaced by the yolk-citrate or milk diluents, the phosphate-yolk diluent has not been so extensively studied. However, since its depressing effect on spermatozoan motility and survival could adversely affect the fertility of highly diluted semen, there is, as pointed out by Salisbury (1957), a need for evidence as to the minimum spermatozoal numbers required for optimum fertility with this diluent before it could be used with high semen dilution rates.

In the early investigations, (Salisbury, Fuller and Willett, 1941; Bratton, Foote, Musgrave and Van Demark, 1949; Stewart, 1950), semen in yolk-phosphate or yolk-citrate diluent gave almost identical conception rates; subsequently Campbell and Edwards (1955) reported that without antibiotics the phosphate diluent gave the best conception rates; these results were reversed when antibiotics were added, since the citrate-yolk was then superior to the phosphate-yolk but the latter was found to be incompatible with the antibiotic used (see page 169). These findings were confirmed by Van Dieten (1957) who, reporting conception rates of 54.5% with yolk-citrate and 50.8% with yolk-phosphate from over 7,000 first inseminations, attributed this difference to the fact that the phosphate buffer was hypotonic, its freezing point depression was -0.431°C. as compared with an optimum of -0.55, and also to an interaction between the phosphate and yolk ions. It is quite possible that the lower semen dilution rates in the early work may account for the different results in the later

work, but the use of an isotonic phosphate buffer with high semen dilution rates has not been studied.

(iii) Citrate-yolk diluent. This was originally described by Salisbury et al. (1941). By giving a clear field, it permits the microscopic assessment of the motility of spermatozoa and it has no adverse effect on semen metabolism. Equal volumes of egg yolk and of either a 3.6 per cent or 2.9 per cent solution of sodium citrate (dihydrate) are used. Although Salisbury et al. (1948) and Aschaffenburg (1950) showed that the latter citrate concentration was isotonic with semen, Melrose and Stewart (1956) obtained equally satisfactory conception rates with equal volumes of either 2.9 per cent or 3.6 per cent sodium citrate solutions and egg yolk. Pursley and Herman (1950) studied the effects of fluids of varying isotonicity on spermatozoal morphology and survival and found that the optimum range of sodium citrate concentrations for survival was 2.3 to 3.5 per cent. Although originally the pH value of the sodium citrate was adjusted to 6.9 prior to the addition of the egg yolk, in practice this is not always carried out, since the addition of the yolk can be depended on to ensure a final pH value of 6.9 to 7.0.

Olbrycht (1947) emphasised that faults in the preparation of this diluent could adversely affect the insemination result. Rikmenspoel (1957) found that, by the use of an ultra-centrifuge, a clear fluid with the protecting properties could be obtained from the yolk-citrate buffer. An apparatus for the filtration of the yolk was previously

described by Hoelzer and Hanske (1952). Although freshly prepared diluent is commonly used, Hurst (1953) observed no affect on fertility after using a yolk-citrate diluent which had been stored frozen for one month after preparation and thawed before use.

(iv) Modified citrate-yolk diluents.

(a) The use of a reduced quantity of yolk has been studied by Almquist (1951,a), by Holt (1952,c) and by Stewart, Melrose and Wilson (1950). All workers reported no effect on fertility when the yolk concentration was reduced to 20 per cent; with further reductions in the yolk content there were, in some cases, adverse effects on fertility, Olds, Oliver, Seath and Carpenter (1951,b) having obtained a significantly lowered conception rate with approximately 16 per cent egg yolk compared with 25 per cent egg yolk.

(b) Whole egg diluents. The inclusion of both the yolk and white of the eggs to give a whole egg-citrate diluent was found by Dunn, Bratton and Henderson (1953,a) to give an insignificantly lower conception rate than the usual yolk-citrate diluent. Similar findings were reported by the Milk Marketing Board, (Report, 1952), and by Hendrikse and Joling (1954). In this diluent it is necessary to add succinylsulphathiozole to bring the pH value down to the optimum level since the egg white has a pH value of 9.0. Apart from the fact that a whole egg diluent can be more easily prepared than a yolk diluent, there is no merit in its use and all workers reported an insignificantly lowered conception rate with it. The precipitation that occurs when yolk citrate buffer is allowed to stand has not been

considered to be of any consequence. However, Stower and Bud-Husaim (1957) reported that with the use of the supernatant fluid from a yolk-citrate-glycine and fructose diluent, which had been allowed to stand for 48 hrs., after the addition of glycerol a satisfactory conception rate could be obtained with semen stored in this for up to 196 hours; the results with a second trial were not so satisfactory with semen stored for over 48 hours and this would require further study.

(v) Other yolk diluents.

(a) Illini-Variable Temperature Diluent (I.V.T.). Following studies of the effect of various diluent constituents and of pH value on spermatozoal survival and metabolism Van Demark and Sharma (1957) described a possible method of storage at room temperature. This diluent consisted of sodium citrate dihydrate (20 g), sodium bicarbonate (2.1 g), potassium chloride (0.4 g), glucose (3.0 g) and sulphanilamide (3.0 g) in distilled water (1 litre); after gassing with CO_2 until the pH value had fallen to 6.3, 1,000 µg streptomycin and 1,000 I.U. penicillin per ml. were added together with egg yolk, the final concentration of the latter being 10 per cent. The semen diluted in this was stored in 1 ml. ampoules in the dark at room temperature. In the preliminary trial a 75.7 per cent conception rate was reported from 111 first inseminations carried out with semen stored at room temperature for up to 7 days compared with 66.9 per cent for 535 first inseminations with semen diluted in egg yolk-citrate and stored up to 3 days at 5°C. However, in subsequent

trials this satisfactory fertility level was apparently not maintained and Van Demark and Bartlett (1958) found it necessary to modify their original formula by the use of higher levels of glucose and egg yolk, by the addition of catalase, and by storage at 40°F. in order to get the best spermatozoal survival rate. Other workers subsequently reported fertility trials with semen stored in this diluent at both room and refrigerator temperatures. Scott and Hardenbrook (1958) by storage at 7°C. for up to 7 days obtained satisfactory results but with only 64 cows; Dunn and Foote (1958) found a marked fall in conception rate with the I.V.T. diluent used at room temperature, on the 1st day this being 69 per cent with 152 first inseminations and falling to 21 per cent with 139 first inseminations on the 6th day after collection; control semen stored for one day in egg yolk citrate at 5°C. had a conception rate of 76 per cent with 963 first inseminations. However, preliminary results reported by McFee, Boyd and Swanson (1958) indicated that a satisfactory conception rate had been obtained with I.V.T. diluted semen stored for up to 3 days compared with semen diluted and stored in an egg yolk-citrate buffer over the same period. The latter showed a higher conception rate on the 2nd and 3rd days when stored in bulk as opposed to the single dose ampoule method used with the I.V.T. diluent. Encouraging results with this technique were also published by Jaskowski, Biwejnis Kłosowska and Wałkowski (1958), who found it beneficial to dilute the semen initially in egg yolk-citrate and later to dilute it further in I.V.T. that contained a reduced antibiotic concentration. The results with I.V.T., over 3 days

storage at 18°C., were above those obtained with the yolk-citrate diluent over the same period. Negative results with this diluent with only 30 cows were obtained by Bonadonna (1958), while Zoder (1958), in spite of having obtained a 71 per cent conception rate with 684 first inseminations with semen stored at room temperature for 7 days, did not consider the diluent to be of any practical value in view of the risk of fungoid growths occurring in it. No such difficulty has been experienced by other workers. Beljakov (1959), using a diluent with a formula apparently similar to that of I.V.T., claimed satisfactory conception rates with semen stored for 4 days. It is worth noting that this worker gassed the diluent with CO_2 at 18°C. after the addition of yolk and semen, and not before it as was the practice with I.V.T. Although there are strong indications that this diluent may be of practical value, it can give variable results and further studies on its formula, pH value and method of preparation would appear to be required.

(b) Glucose and fructose additions with and without sodium bicarbonate. Although glucose was a constituent of the original semen diluents (Anderson, 1945), no indications were given as to its effect on fertility. Salisbury and Van Demark (1945) demonstrated that the addition of glucose prolonged the life of the spermatozoa in vitro, and also stimulated the rate of glycolysis even when there was no shortage of glucose in the storage medium. Valerani (1948) confirmed this improved motility effect and found no adverse effect on fertility with a glucose addition. However, the France Soc. Cooperative D'Insemination Artificielle de Picardie (Report, 1953,c), in a

study of the inclusion of glucose in the yolk-citrate diluent in order to prevent spermatozoal agglutination, obtained the best results with isotonic levels of both glucose and sodium citrate, but conception rates with this were lower than with skim milk. The extensive fertility data published by Ohms and Willett (1958) showed that with the inclusion of glucose in a yolk-citrate diluent the 1st day non-return (60 - 90 day) conception rate was 67.8 per cent compared with 66.0 per cent with yolk-citrate without glucose. On the 2nd day of storage the respective conception rates were 55.7 per cent and 61.7, thus indicating a significant reduction in fertility of semen stored in the glucose yolk-citrate diluent.

The use of glucose to maintain the required osmotic tension in diluents was extensively reported on by Kampschmidt, Mayer, Herman and Dickerson (1951) and by Kampschmidt, Mayer and Herman (1953,b). The required pH value and maximum motility and survival ratings were obtained with one volume of egg yolk added to five volumes of a mixture containing 1 part of a 1.3 per cent sodium bicarbonate solution and 4 parts of a 5 per cent glucose solution. These observations on spermatozoal survival were confirmed by Smith et al. (1954) and by Dimitropoulos (1954), but the latter noted that the samples from individual bulls gave variable results. However, Willett and Ohms (1957,a), although confirming the foregoing results with semen at a dilution rate of 1:9 (180 million spermatozoa per ml.), also reported that at high dilution rates (i.e. spermatozoal concentrations less than 15 million per ml.), the survival in yolk-glucose-

bicarbonate was lower than that in yolk-citrate unless sulphanilamide was included in the bicarbonate diluent. These authors found that after storage for 10 days in the yolk-glucose-bicarbonate with the low spermatozoal concentration the pH value rose to 7.69, whereas it fell to 6.81 with a dilution level of 1:9; they postulated that the metabolism of the spermatozoa in low concentration was stimulated by the high pH value of the diluent and that sulphanilamide acted by preventing this effect. Although in the fertility studies reported by Senegacnik (1956) a high overall conception rate was obtained with Kampschmidt's diluent, Melrose and Stewart (1956) found no overall beneficial effect. In fact a marked depressing effect on conception rate on the third day of use of the semen, when compared with the standard yolk-citrate buffer, was obtained with Kampschmidt's buffer without sulphanilamide. The dilution rate of 1 part semen in 50 parts diluent was constant in both diluents. More recently, Foote, Young and Dunn (1958) investigated the use of semen in a diluent containing egg yolk, sodium citrate, potassium chloride, glucose and glycine (referred to as C.U.E.), and they found this to be as effective as yolk-citrate over a 2 day storage period. The C.U.E. diluent owing to its many constituents requires careful preparation, and for this reason it may have a limited routine use, but more extensive fertility studies are required. Attention has also been paid to the possible inclusion of fructose in diluents. Pérez Y Pérez (1954,b) reported that in a buffered saline diluent fructose was superior to glucose in promoting spermatozoal survival, but in

non-buffered saline these results were reversed. If these findings were due to the buffering capacity of the saline they are not in accord with the subsequent report by Sergin (1956) who claimed that the viability in fructose-yolk-citrate was better than that in glucose-yolk-citrate and that less lactic acid was formed from the metabolism of fructose than of glucose. However, Kok and Van Dieten (1957), in an extensive controlled field investigation involving 16,000 inseminations, found no beneficial effect on fertility with the addition of 10g. fructose per litre of diluent, but a beneficial effect on spermatozoal survival was also noticed by these authors. Melrose and Stewart (1956) had previously reported negative results with the addition to yolk-citrate of a much lower concentration of fructose (0.5 g./litre), it having been calculated that this addition, together with the amount present in the semen at time of collection, would provide sufficient fructose for the metabolic requirements of the spermatozoa.

The addition of glucose and/or fructose has not been clearly shown to improve conception rates, although there is a beneficial effect on motility under certain conditions. The evidence indicates that the additions of these carbohydrates benefit the spermatozoa by maintaining the correct osmotic tension and electrolyte balance rather than by increasing the concentration of utilisable carbohydrate.

(vi) Enzyme, hormone and vitamin additions.

(a) Mucinase. Following the observation that the spermatozoal survival in the female genital tract decreased in proportion to the increased viscosity of the vaginal mucus, and since the amount of the

latter is increased at oestrus, Sokolovskaja (1950) postulated that the addition of mucinase would increase the chances of conception by reducing the viscosity of the vaginal mucus. A small trial, carried out by Aslanjan (1953) in which 276 cows were used, suggested that this might be so. More recently, however, and again with limited data, Sokolovskaja, Drozdova, Golyseva, Korotkov, Maksimov and Lebedeva (1956) indicated it was preferable to add antibiotics and glycerol along with the mucinase; the benefit of the latter would not appear to have been clearly confirmed.

(b) Catalase. Although the work of Tosic and Walton (1950) indicated that catalase would prevent the spermicidal action of hydrogen peroxide, which could accumulate in semen stored in egg yolk diluents, Van Demark et al. (1950) were unable to demonstrate any beneficial effect on either conception rate or spermatozoal survival with the addition of 1 part Vitazyme Catalase (Sarrett) to 10,000 parts of a citrate-yolk-sulphanilamide diluent.

(c) Hyaluronidase, from bull testes, was added by Johnston and Mixner (1951) at the rate of 5 - 40 mg/ml. of semen diluted in yolk-citrate; the similar addition of 2 - 8 mg. hyaluronic acid did not affect motility but higher dosages were toxic to spermatozoa. In controlled fertility trials by Fiorentino and Caretta (1951) an improved conception rate in 3 out of 4 bulls was reported with the addition of crystalline hyaluronidase (0.1 g/10 ml. semen) but, although there was no deleterious effect with this addition, the authors attributed the increased conception rate to some unknown

factor. However, Pérez Y Pérez (1954,a) reported that the hyaluronidase addition (40 - 80 mg%) to the diluent increased the numbers of abnormal spermatozoa and shortened spermatozoal survival; vitamin C added along with hyaluronidase was found to give similar results.

(d) Antistin was claimed by Orlowski (1955) to counteract the harmful effect of histamine and to improve the spermatozoal survival when added to diluted semen.

(e) Thyroxine. Schultze and Davis (1949) obtained an increase (up to 6.7 per cent) in conception rate with semen diluted in yolk-phosphate buffer containing D-L-Thyroxin and used for 2,289 inseminations; the increase in fertility with the treated semen was greatest after storage for 2 to 4 days. However, Swanson and Boatman (1953), in spermatozoal motility studies, reported negative results after adding thyroxin to the semen of two bulls in which mild hypothyroidism had been induced by feeding thiouracil. The use of this in diluents has not been extensively studied.

(f) Oestrogen and progesterone. Although oestrogenic activity was demonstrated in bull semen extracts by Schaffenburg and McCullagh (1956), the possible function of this remains unknown. The addition of oestradiol at levels up to 1 µg per ml. diluent had no effect on fertility in a controlled fertility trial involving 1,441 cows (Reading Cattle Breeding Centre; Report, 1960,b). Staples, Hansel, Foote and Dunn (1958) were also unable to demonstrate any effect on fertility with separate or combined addition of oestradiol and progesterone to

diluted semen. Oestradiol at levels of up to 200 µg/ml. and progesterone up to 5,000 µg/ml. diluted semen were used. The addition of dienoestrol to diluted bull semen was reported by Pérez Y Pérez (1956) to reduce both the length of spermatozoal survival and also the proportion of abnormal spermatozoa; a further study by Pérez Y Pérez (1957) indicated that the addition of 500 I.U. dienoestrol to 4 ml. semen increased the motility rating over the next 48 hours, but thereafter it was lower than in the control samples; no fertility trials were reported with this addition.

(g) Vitamin B12. Although Busch (1954) obtained inconclusive results based on viability tests at different temperatures with the addition of varying concentrations of Vitamin B12 to 26 ejaculates from 10 bulls, there was an indication of improved spermatozoal survival at storage temperatures of 18° to 32°C. but not at 4° to 6°C. No metabolic studies were made on this semen.

(h) Vitamin E. In a limited fertility trial carried out when Vitamin E deficiency could not be expected, Schlaak (1956) reported pregnancy rates of 58.5 per cent in 309 cows inseminated with Vitamin E treated semen and 57.3 per cent with 152 cows inseminated with untreated semen. When this trial was repeated when Vitamin E deficiency was expected, the results were 51.4 per cent of 72 cows pregnant following insemination with treated semen and 39 per cent of 182 cows pregnant after insemination with untreated semen. There was no definite evidence that there was a Vitamin E deficiency in the last group, but if there were, these results indicate a possible

beneficial effect with this addition under certain conditions.

These additions to diluents have not been investigated in extensive fertility trials. Since the effects of these additives may differ with individual bulls, it would appear that the overall benefit, if any, will not be readily determined.

(vii) Other diluents

(a) Coconut milk. The use of this for storage of semen at room temperature has been investigated by Norman, Johnson and Porterfield (1958,a). Semen that was stored in equal volumes of coconut milk and a 4.32 per cent sodium citrate solution plus 100 mg per cent calcium carbonate, 80 mg per cent penicillin and 90 mg per cent streptomycin at room temperature for 6 days could be re-activated by resuspending in some fresh diluent or by a further addition of calcium carbonate. Using semen from 4 bulls at a low dilution, Chieffi and Masotti (1959,a) reported that on storage at 2° to 5°C. a coconut milk/egg yolk mixture gave a better survival rate than did the yolk-citrate diluent.

(b) Tomato juice. Motility studies by Chieffi and Masotti (1959,b) showed a satisfactory spermatozoal survival rate in semen contained in a diluent consisting of 3 parts tomato juice : 1 part egg yolk.

(c) Honey. Although honey (10 per cent) in distilled water was reported by Pecnikov and Skatkin (1955) to be beneficial for stallion semen storage, there are no reports of investigations with bull semen.

(viii) The use of certain inorganic salts and low molecular weight organic compounds in diluents.

(a) Gelatin. The phosphate-glucose-gelatin mixture, described originally by Milovanov (1938), was subsequently used by Sørensen (1946) with the addition of 10 per cent egg yolk. The semen dilutions were carried out at 35°C. the bulk of the diluted semen being divided into single doses in paraffin coated straws, allowed to cool for storage, the spermatozoa thus becoming suspended in solidified gelatin. The inseminations were performed by using a special instrument (see page 227). Although this technique was extensively used in Denmark, Rottensten and Wibling (1951), in a comparison of this gelatinous diluent with the fluid citrate-glucose-sulphanilamide diluent over 4,200 first and repeat inseminations, found a higher overall conception rate with the latter diluent but only the conception rate difference of 4.6 per cent with first inseminations was significant. Hoelzer (1948) observed a reduction in spermatozoal motility with the addition of 5 per cent gelatin to glucose phosphate, and also the addition of yolk gave results inconsistent with other reports. Schmidt (1955) found no marked beneficial effect of the addition of 1 per cent gelatin to yolk-phosphate, to yolk-citrate or to milk diluents. With yolk diluents there is, therefore, no definite indication of any benefit following the inclusion of gelatin.

(b) Glycerol. In addition to its protective physical action glycerol has also been shown to be metabolised by spermatozoa

(White, Blackshaw and Emmens, 1954; O'Dell, Flipse and Almquist, 1956 and White, 1957). However, O'Dell, Almquist and Flipse (1959 b,d) showed that the glycerol uptake was less with washed semen diluted in yolk-citrate or milk than with that diluted in physiological saline; also glycerol inhibited lactic acid production in semen diluted with these commonly used diluents, thus indicating its possible usefulness in the prolonged storage of semen at 5°C. Pickett and Merilan (1959) found that a combination of 1 per cent glycerol and 1 per cent fructose gave a longer spermatozoal survival at 38°C. than either glycerol or fructose alone. A similar depressant action on fructolysis with a 1 per cent glycerol - 1.25 per cent fructose combination was reported by O'Dell, Almquist and Flipse (1959,c). The fertility studies with glycerol containing diluents will be discussed later (see page 136,195).

(c) Sodium salicylate. Schindler and Volcani (1953) reported that spermatozoal survival was improved by the addition of 0.5 per cent of sodium salicylate to yolk-citrate compared with the addition of sodium benzoate or sulphanilamide to the diluent.

(d) Sodium carbonate. Following the use of this for the activation of semen diluted in yolk-citrate plus antibiotics, Rickard, Ludwick, Hess and Ely (1957) found a significant improvement in conception rate with semen used on the 2nd day after collection. There was an insignificant difference on the first day of use when compared with control semen diluted in egg yolk citrate.

(e) Sodium chloride. The use of physiological saline as a diluent was reported by Knaller (1950) to have no deleterious effect on conception rate, but the spermatozoal survival rate was lower in a saline than in an egg yolk diluent. Salisbury (1957) pointed out that although an 0.9 per cent sodium chloride diluent stimulated spermatozoal respiration, it would only be a satisfactory diluent in the presence of oxygen. It had no buffering capacity and the spermatozoa, in the absence of oxygen, would be unable to withstand a lactic acid accumulation with consequent lowering of the pH value. This postulation was confirmed in the reported fertility data, which showed that, with stored semen, a sodium chloride-egg yolk diluent resulted in a lowered conception rate compared with the usual yolk-citrate diluent. Rose and Maupoumé (1957) have reported limited fertility data on the use of filtered and subsequently diluted sea water to replace sodium citrate in the yolk-citrate diluent.

(f) Blood serum, seminal plasma and blood plasma substitutes. Albright, Ehlers and Erb (1957), reported that concentrated spermatozoal samples, showing little or no motility after 2 to 4 hours incubation at 37°C., could be stimulated by the addition of seminal plasma. No unfavourable, and even a beneficial, effect on motility following the suspension of bull spermatozoa in stallion seminal plasma was noticed by Olbrychtowa and Walkowski (1959). Kriisa (1947) reported a reduction in the fertility of bull spermatozoa diluted with inactivated mare serum, whilst Schmidt and Kroll (1953), in a study of the effect of various diluents, reported decreased spermatozoal survival

with inactivated cattle blood serum. However, Asher and Kaemmerer (1950) claimed a spermatozoal survival as good as in glucose phosphate with neutralised "Periston" (3.5 per cent solution of polyvinylpyrrolidone), but the survival rates were relatively short and the use of such blood plasma substitutes for semen storage has not been reported elsewhere.

In general, therefore, except for the 0.9 per cent solution of sodium chloride which appeared to stimulate the aerobic metabolism, none of the above additions had any marked effect on spermatozoal survival, and only with glycerol and sodium carbonate were any beneficial effects on fertility recorded.

(ix) Glycine containing diluents. Knoop and Krauss (1944) reported an improved survival with the addition to the phosphate/yolk diluent of 1.09 per cent glycine, when compared with yolk/gelatin and yolk phosphate diluents. Prolonged spermatozoal survival with the use of a glycine containing salt diluent was obtained with storage at 38°C. but not at 4°C. when compared with the yolk-citrate diluents (Tyler and Tanabe, 1952). Later Roy and Bishop (1954) reported that there was longer spermatozoal survival in a diluent consisting of equal volumes of egg yolk and a 4 per cent glycine solution in distilled water at 5°C. than in the yolk-phosphate or yolk-citrate diluents. Beneficial effects on spermatozoal survival were also reported by Rakes and Stallcup (1956), Baier, Leidl and Greiff (1957) and Saha and Sing (1958), but when Strom (1956) compared yolk-glycine with the yolk-citrate diluent in a split sample trial, involving 3,000 inseminations, no improvement in the overall fertility could be demonstrated. Semen in the yolk-citrate diluent gave an

insignificantly higher conception rate than that in yolk-glycine after storage for 50 hours or more. Similar results with fertility studies with milk-glycine diluents were reported from Reading Cattle Breeding Centre (Report, 1958). Adler and Rasbech (1956,c), in a controlled investigation with 8,000 inseminations, obtained 60 to 90 day conception rates of 66.2 per cent and 66.0 per cent with semen diluted in yolk-citrate and glycine-fructose-yolk diluents, respectively, and used within 12 hours of collection. In an uncontrolled observation, a 27.5 per cent conception rate was reported with 400 inseminations with semen stored in the glycine diluent for 7 days at 4°C. With bull semen, therefore, in spite of the beneficial effect on survival, there is no evidence of an improvement on fertility with the inclusion of glycine in the diluent. The observations made on changes in pH value of glycine containing diluted semen by Rakes and Stallcup (1956) indicated that glycine does not buffer the diluted semen as well as does sodium citrate.

(x) Milk diluents. Phillips and Lardy (1940) reported that bull spermatozoa could survive in an evaporated milk diluent for 48 hours but that they were immotile after 72 hours, and Underbjerg, Davis and Spangler, (1942) concluded that autoclaved milk had no beneficial effect on fertility. There were encouraging reports by Mihailov (1949) on the use of milk as a stallion semen diluent. After a series of investigations into various diluents, Jacquet (1951) recommended the use of canned skim milk since, when this was compared with the usual egg yolk-citrate buffer at over 26 insemination centres, a 9 to 15 per cent increase in conception rate was obtained;

the canned skim milk was also considerably cheaper. However, Jacquet and Cassou (1952,a,b) subsequently realised that the canned product was liable to vary in quality and they advised that, in its stead, a 10 per cent skim milk powder diluent containing 10 per cent egg yolk together with antibiotic additions should be used; when compared with egg yolk citrate this milk/yolk resulted in an improved conception rate, which was most marked with low fertility bulls. No reference was made to any variations in the powdered product or to the method of preparation of the diluent. Around this time, Thacker and Almquist (1951) reported that a satisfactory spermatozoal survival rate could only be obtained in either homogenised milk or pasteurised skim milk after these had been boiled for at least 10 minutes in a covered vessel and allowed to cool prior to the addition of the semen. Sanfile (1953) claimed a 4.2 per cent higher conception rate with heated skim milk than with egg yolk-citrate. Weiss (1952) reported that skim milk plus 2 per cent egg yolk compared favourably with the usual yolk-citrate diluent, but the tests carried out by the Milk Marketing Board (Report 1952) indicated that a boiled milk diluent was inferior to yolk-citrate buffer. Other workers, having realised that milk diluents could have economic and practical advantages, reported their investigations into the preparation of milk diluents. Also a possible usefulness of the various milk products either alone or in combination with other additives, such as egg yolk, glycerol and glycine, was indicated. Albright, Ehlers and Erb (1956), basing their conclusions on motility studies, claimed that both washed and unwashed

spermatozoa were capable, under certain conditions, of utilising certain constituents of milk as a source of exogenous energy.

(a) Heat and/or chemical treatment of milk for use in diluents. Investigations into the spermicidal action of unheated milk were reported from the U.S.A. by Thacker and Almquist (1953), who showed that gentle boiling of milk resulted in better spermatozoal survival rates than did vigorous boiling; also heating the milk by direct heat in an open vessel at boiling point for more than 10 minutes reduced spermatozoal survival, whereas this was improved if the milk was heated at 92° - 95°C. for aslong as one hour in a double boiler. Subsequently, Thacker, Flipse and Almquist (1954) showed that the spermicidal action was associated with the albumen containing fraction of the milk and, as a result of their spermatozoal survival studies with this albumen fraction, postulated that the heating of milk inactivated or destroyed a factor toxic to the spermatozoa, but this treatment also produced a factor which reduced spermatozoal survival. Heating thus could give rise to the production or setting free of two factors having opposite effects on spermatozoal survival. These findings also suggested that, in view of possible variations in the manufacturing processes, care must be exercised in the use of milk products in diluents. This was reported on further by Flipse, Patton and Almquist (1954), who indicated that the toxic factor present in unheated milk was probably lactenin, an antistreptococcal substance normally present in the albumen fraction of milk. This could be reliably inactivated

by controlled heating.

Boyd, Perkins, Olds and Seath (1954) and Johnson, Flipse and Almquist (1955) demonstrated that the toxic factor in unheated milk could also be inactivated by the addition of sulphydryl compounds to the unheated milk diluent; this addition was shown to result in normal spermatozoal survival *in vitro*. These authors confirmed that the major spermicidal factor in fresh milk, thought to be lactenin, could be inactivated by sulphydryl groups, which were either released through the denaturation by heating of the B-lactoglobulins (in the albumen fraction of the milk) or which could be added to semen direct as cysteine hydrochloride or glutathione. Cysteine hydrochloride at a level of 1 mg/ml. unheated diluent was found to give the best survival rates. However, the suggestion that the chemical treatement of the unheated milk would be preferable to the heat treatment, since it eliminated the risk of over-heating, cannot be accepted without more knowledge of the chemical changes involved. Moreover, the heat treatment does kill off pathogenic or contaminant organisms, which could be present in the milk. The work of Saacke, Almquist and Flipse (1956) indicated that, owing to variations in the resistance of their proteins to denaturation, different batches of skim milk might require different degrees of heat treatment. From the 4 batches tested, it was concluded that optimum spermatozoal survival could be obtained with heating for 1 minute at temperatures ranging from 87° to 97°C. or for 10 minutes at 77° to 97°C. For practical purposes a narrower temperature range would appear to be advisable, e.g. 92°C. for

10 minutes, heating being done in a water bath. Melrose, Stewart and Bruce (1958) stressed the need for careful control of this heating and for ensuring glassware that had previously contained unheated milk was not subsequently used for heated milk unless it had been cleaned and sterilised. Laboratory tests had shown that the addition of 0.6 ml. unheated to 10 ml. heated milk was detrimental to spermatozoal survival.

(b) Field results with different milk diluents. Since controlled fertility trials are the only reliable methods of assessing the practical value of any of the diluent combinations, the main findings have been summarised in Tables 6 and 7. (pages 140 to 143).

(1) Homogenised milk. Whole milk can only be used for routine inseminations if it is homogenised. Some early work was, however, carried out with it untreated. The results, quoted in Table 6, indicate that the overall conception rates with homogenised milk were equal to, or superior to, those with egg yolk citrate. The bulk of the inseminations were performed either on the day of collection or on the following day (U.S.A. reports chiefly), but the report by Perkins, Carpenter and Seath (1955) showed a marked lowering of fertility with semen in the homogenised milk used on the 3rd day. Flerchinger, Erb and Ehlers (1953) reported a 6 per cent difference between 1st and 3rd day conception rates with both diluents, Almquist , Flipse and Thacker (1954), with limited data, found no drop in the 3rd day conception rate with either diluent; in a previous study these authors had excluded all results obtained with semen used later

than the first day after collection. Perkins et al. (1955) also remarked that of the 31 bulls in their investigation, 14 had an improved conception rate with the milk, 15 were better with the egg-yolk citrate, whilst 2 were the same in both diluents; in other reports it was rare to find bulls with conception rates lower with the milk than with the yolk-citrate diluents. In this and in the investigations described by Almquist (1954) and by Dreher and Webb (1953) the bulls of lowest fertility with the yolk diluent showed an increased conception rate in the milk. This finding has been discussed later (see page 177).

Almquist et al. (1954) found no beneficial effect by increasing the viscosity of the milk with corn starch whilst Kerruish (1956) reported a reduced viability of the spermatozoa in the homogenised milk, which also deteriorated on storage on certain occasions. From the evidence available, it can be concluded that homogenised milk, after carefully controlled heat treatment, can be reliably used as a diluent for semen stored up to one day after collection (i.e. up to and including the day after collection), but further fertility data are required on the effects of longer storage. Mihailov (1957) reported favourably with limited data on the use of boiled cow milk, while Macpherson (1957) also claimed an improved spermatozoal survival rate in goat milk.

In whole milk diluents, owing to their opacity, assessment of spermatozoal motility cannot be reliably made with an ordinary microscope, but Zakrewska (1956) reported on the use of phase contrast

microscopy to overcome this difficulty.

(2) Skim milk. Since this is considerably cheaper, its use has appealed to many commercial organisations. The findings, summarised in Table 7, show that heat treated, fresh skim milk was as effective as yolk-citrate except in the report of Bolton and Durrell (1954) who suggested that the reduced conception rate with the milk may have been due to the higher degree of bacterial contamination which they had found to exist in the milk, in spite of its having been heated to 96° to 100°C. Almquist et al. (1954) found that with the skim milk the conception rates were slightly, but insignificantly, higher than with the homogenised milk. In vitro tests with skim milk powder were reported by Collins (1953) and by Marion and Olson (1952) who indicated that the survival in diluents, prepared from this without heating, reflected the degree of heating in their original manufacture. Fertility trials with a powdered skim milk diluent were reported by Melrose (1956) who found this to be superior to egg yolk-citrate since a significant rise in conception rate was obtained in 6 of the 17 bulls used. This increase, which occurred in the bulls with the lowest conception rates with the egg yolk-citrate, was in accord with the findings of Dreher and Webb (1953), Perkins et al. (1955) and Almquist (1954) reported above. Melrose et al. (1958) found powdered skim milk to be as effective as fresh skim milk as a diluent but also reported variable results with in vitro tests with unheated skim milk powder diluents, again confirming the effect of the manufacturing process. Furthermore,

in their investigations there were, on occasions, inexplicable differences between the 1st and 2nd day conception rates; the increased efficiency of the antibiotic addition in the skim milk diluent was indicated and the need for using a milk powder from a reliable source was stressed. The adverse effects on spermatozoal survival of diluents prepared from fortified skim milk products were referred to by Saacke, Almquist and Patton (1955). By using a skim milk powder a centre can, however, obtain a standard diluent for use over a given period, and there is also no risk of introducing infection to the centre, which could occur when fresh skim milk is used.

(3) Milk/yolk diluents. Extensive fertility trials with this have not been published, although this diluent is now widely used. Peters (1953), Fiorentino (1954), and Zebracka-Szczesna (1957) have reported improved survival rates with the addition of 10 per cent yolk to milk. Improved fertility rates with this were claimed by Kluza, Michalska, Zebracka-Szczesna and Schmidt (1958). The increased birth weight of calves, and higher percentage of male calves associated with the use of this diluent by Sobek (1953) has not been confirmed elsewhere. The increased fertility with milk/yolk over yolk-citrate diluents, reported by Bonadonna (1954), could not be definitely attributed to the milk-yolk diluent since the two diluents were used separately and at different times of the year.

Following motility studies that demonstrated a beneficial effect with the addition of 5 per cent yolk to skim milk diluents, Hendrikse and Joling (1957) reported consistently higher conception

rates with milk/yolk diluents than with whole egg yolk citrate diluent. This controlled trial involved over 14,000 inseminations and the reported conception rate increases were 1.4 per cent, 3.1 per cent and 2.2 per cent with first, repeat and all inseminations, respectively. These conception rate increases were most marked with 3rd and 4th day semen, and the findings also differed from those previously reported in that some bulls had an improved fertility in the whole egg yolk citrate, whereas others showed this in the milk/yolk. At the Reading Cattle Breeding Centre, (Report, 1960,b) a trial, involving 5,786 first inseminations to compare a diluent containing 9 per cent skim milk powder (wt/vol.) and 12.5 per cent egg yolk in distilled water with the usual egg yolk-citrate diluent, gave overall 112 day non-return conception rates of 61.7 per cent and 59.2 per cent respectively with semen used over 3 days; the third day conception rates were 61.0 per cent in milk/yolk with 940 first inseminations and 52.8 per cent in yolk-citrate with 994 first inseminations. The Milk Marketing Board (Report, 1959,a) reported a 69.3 per cent conception rate with a skim milk powder diluent containing 5 per cent egg yolk; with fresh skim milk over the previous year the conception rate was 69.0 per cent.

(4) Cream. Scandinavian workers have studied this with conflicting results. Adler and Rasbech (1956,b) used the homogenised sterilised 9 per cent export cream as a diluent and obtained a conception rate of 69.4 per cent with 2,931 first inseminations (cf. 63.6 per cent with 2,370 first inseminations with egg yolk-citrate and 69.2 per cent with 2263 first inseminations with heated skim milk).

In contrast, Aamdal (1956) reported a lowered conception rate when cream was compared with yolk-citrate.

(5) Combination of milk with glycine and/or glycerol.

The increasing demand for a diluent that would enable semen to be stored at normal refrigerator temperatures and to have a satisfactory fertility level after several days (4 - 6 days) prompted the attention to the possible use of glycerol and/or glycine along with milk for this purpose. Flipse and Almquist (1956) reported that spermatozoal survival in equal volumes of heated fresh skim milk and 0.5 M. glycine was equal to that in yolk-glycine and inferior to that in heated skim milk alone. Similar results were obtained with skim milk powder, but with this the survival rates were further improved by the addition of either fructose (0.2 per cent) or glycerol (5 per cent). In vitro results, published by Albright, Ehlers and Erb (1958,a), were also in accord with these reports. However, at the Reading Cattle Breeding Centre (Report, 1960,b) no beneficial effect on fertility could be demonstrated by the addition of either glycerol or glycine/glycerol to the skim milk powder diluent. Most attention has, however, been directed to the use of glycerol alone in milk since Almquist (1957) claimed an extended fertilisable life of semen diluted in milk plus 10 per cent glycerol. Ohms and Willett (1957) when using glycerol in a skim milk powder diluent obtained a markedly reduced fertility compared with the yolk-citrate control over 2 days of use, but the skim milk powder diluent alone showed a lower conception rate than the yolk-citrate in this study. Williams, Green and Dombroske (1957) compared heated homogenised milk with and without the addition of 10 per cent glycerol and demon-

strated a highly significant increased conception rate with the milk-glycerol diluent on the 2nd and 3rd days of use. In a similar study O'Connor and Smith (1959) using skim-milk and skim milk glycerol diluents obtained, with the latter, conception rate increases of 4.7 per cent and 10.6 per cent with semen used on the second and third day after collection, respectively.

Almquist (1959), reporting more extensively on this glycerol addition, has shown that the glycerol must be added after the semen, diluted initially in milk alone, has been cooled to 5°C. The results reported with this procedure would appear to warrant its extensive trial, since with the milk glycerol diluent the conception rate was 5 per cent higher than that with the milk alone.

In general, both whole and skim milk diluents by virtue of their cheapness can be safely used in routine artificial insemination for semen used up to 36 hours after its collection. Careful control of the necessary heating process must be exercised, and when a skim milk powder is used it must be obtained from a reliable source. The addition of sulphydryl containing compounds, instead of heating, would appear to be as effective in promoting spermatozoal survival but, without heating, there is a greater risk of bacteria being present and spreading through the diluted semen. These are also indications that the inclusion of up to 10 per cent yolk in the milk diluent may improve its efficiency.

(xi) Commercial diluents. In these, the aim has been to replace

the egg yolk by simpler and less expensive materials. Phillips and Spitzer (1946), after investigating the use of blood fibrin, lecithin, various gum products, glucose and galactose and egg yolk, concluded that a synthetic pabulum should contain freshly purified lipids, glucose, a buffer, gum and either sulphathalidone, sulphasuxidine or streptomycin. Subsequently, semen diluted in a buffer with these constituents was reported by Bayley, Cobbs, and Barrett (1950) to have a significantly lower conception rate than that used in the normal egg yolk-citrate. In the U.S.A. the Ortho buffer was favourably reported on by Hurst and La Master (1948); Bratton et al. (1949) also obtained conception rates with this comparable to those with the conventional citrate- and phosphate-yolk diluents containing sulphanilamide. In Europe several commercial diluents have been referred to in the literature but the exact composition of these is not always disclosed. Kust and Jurgens (1951) and Weiss (1951) reported that spermatozoal survival in a diluent, named SPV 161, was as good as in yolk-citrate; Aehnelt (1951) failed to confirm this report and claimed that Spermasol — 20 per cent egg yolk gave the best survival rates. Hoelzer (1951), in an uncontrolled observation, reported satisfactory conception rates with Spermasol, which has as its basis sodium citrate, potassium phosphate, gelatin and streptomycin, 10 to 30 per cent egg yolk being added just before use. Kobert (1952) found that semen, diluted in Spermasol and used for insemination after 24 hours' storage, had a higher conception rate than that in yolk-citrate. Bonfert (1953) claimed Spermasol to be superior to other commercial diluents and later Bonfert (1956,a) reported

satisfactory conception rates with what would appear to be two modifications of this referred to as Spermasol N and Spermasol S. Recently, however, Krawarik (1957) found that spermatozoal survival in Spermasol + glycine (Spermasol G.T.) was inferior to that in egg yolk-citrate.

Ullner (1953) had previously reported that spermatozoal survival in Spermasol yolk was equal to that in yolk-citrate and also in another diluent, Dilopten, after 6 to 8 days' storage. Dilopten, which is yolk free and contains glucose, sodium citrate, a gelling agent and phosphatides of animal origin, was also favourably reported by Spies (1953), who, with over 1,000 inseminations, obtained similar conception rates with this and with the yolk-citrate diluent. Prolonged spermatozoal survival in an improved type of yolk diluent, Seminan, was claimed by Nishikawa, Waide and Otsuki (1955). This diluent contained 25 per cent egg yolk, 1.6 per cent sodium citrate, 0.11 per cent potassium citrate, 0.15 per cent sodium phosphate, 0.97 per cent glucose, 0.1 per cent homosulphamine and 0.05 per cent sodium sulphamesadin in distilled water, the semen diluted in this being stored in sealed ampoules at 4°C. but no extensive fertility results with this were given.

There is little published evidence to show that any of the above synthetic diluents are being used, but a diluent of standard composition could be a distinct advantage in carrying out controlled investigations and could also prove to be useful at a small-scale insemination station with limited facilities for preparation of diluents.

TABLE 6

COMPARISON OF HOMOGENISED MILK AND YOLK DILUENTS

Ref.	Diluents and treatment	Number of First Insem-inations	Non-return Conception Rate Per Cent	Remarks
Thacker and Almquist (1953)	Homogenised milk-boiled/no antibiotics	2,381	72.7	Relatively fertile bulls used.
	Yolk-citrate plus antibiotics	2,620	71.4	
Perkins et al. (1955)	Homogenised milk - heated	10,012	69.4	14 bulls gave highest conception rate in milk and 15 bulls highest in yolk-citrate; 2 bulls had equal fertility with both diluents.
	Yolk-citrate	9,927	70.3	
Almquist et al. (1954)	Homogenised milk - heated plus anti-biotics	1,570	76.1	Average and high fert-ility bulls used.
	Yolk-citrate plus antibiotics	1,565	75.9	
Almquist (1954)	Homogenised milk-heated plus antibiotics	4,178	71.6	Low fertility bulls showed increased conception rate in milk.
	Yolk-citrate plus antibiotics	4,222	64.2*	

Ref.	Diluents and treatment	Number of First Insemin-ations	Non-return Conception Rate Per Cent	Remarks
Dreher and Webb (1953)	Homogenised milk-heated plus antibiotics	6,009	69.8	Increased conception rate with milk occurred with low fertility bulls.
	Yolk-phosphate plus antibiotics	6,060	59.2*	
Kerruish (1956)	Homogenised milk - heated	4,637	68.8	90-120 day non-return conception rate.
	Yolk-citrate	5,051	69.9	

All conception rates were on a 60-90 day non-return basis except where indicated otherwise.

* Conception rate differences were statistically significant.

TABLE 7

COMPARISON OF SKIM MILK AND YOLK DILUENTS

Ref.	Diluents and treatment	Number of First Insem-inations	Non-return Conception Rate Per cent	Remarks
Almquist et al. (1954)	Skim milk - heated	2,873	67.8	Average and high fertility bulls used.
	Yolk-citrate	2,674	66.8	
Kerruish (1956)	Skim milk - heated	6,850	70.7	90-120 day non-return conception rates.
	Yolk-citrate	7,978	69.1	
Bolton and Durrell (1954)	Skim milk - heated	1,494	66.4*	Suggested reduced conception rate in milk due to bacterial contaminants.
	Yolk-citrate	1,555	70.7	
Adler and Rasbech (1956,b)	Skim milk - heated	2,263	69.2	
	Cream-homogenised and sterilised	2,931	69.4	
	Yolk-citrate	2,370	63.6*	
Melrose (1956)	9 per cent skim milk powder-heated	5,076	68.4	Conception rate increase confirmed to 6 of 17 bulls used. 112 day non-return conception rates.
	Yolk-citrate	5,113	63.5*	

Ref.	Diluents and treatment	Number of First Insemi-nations	Non-return Conception Rate Per cent	Remarks
Melrose et al. (1958)	9 per cent skim milk powder-heated	2,025	67.3	112 day non-return conception rates.
	Fresh skim milk-heated	1,949	68.9	

Note:

1. Antibiotics were included in all diluents.
2. Except when indicated otherwise, all conception rates were 60-90 day non-return figures.
3. Conception rate differences which were statistically significant are marked thus *.

S E C T I O N VII

SEMEN HANDLING, COOLING, DILUTION AND TRANSPORT

As indicated earlier the first essential in semen handling is to avoid damage to the spermatozoa by temperature shock, (Chang and Walton, 1940; Salisbury, 1941; Easley, Mayer and Bogart, 1942), the basic procedure being to store the semen at 4° to 6°C. at the optimum dilution rate consistent with maintenance of the fertilising capacity of the spermatozoa. The diluent, whilst enabling the semen to be used for a large number of cows, has also a protective action (see page 104) but it is important to add it to the fresh semen as soon as possible after collection. The full dilution may be done initially, prior to cooling; alternatively, a preliminary low dilution may be made to enable the cooling measures to be undertaken, pending the estimation of the spermatozoal density of the sample, which can then be later diluted to its final extent with diluent at the same temperature to give the required number of spermatozoa per insemination dose. Modifications of the various stages involved have been extensively studied and will, therefore, be considered separately.

A. Dilution rate and optimum spermatozoal numbers required per insemination

The many studies of the effect of dilution rates on fertility have been of immediate practical value in artificial insemination operations (see table 8). Salisbury, Beck, Cupps and Elliott (1943,a)

TABLE 8

DILUTION RATE STUDIES

Ref.	Dilution Rate	Age of semen (1)	Number of inseminations	Conception Rate (2) per cent	Remarks
Salisbury, Elliott and Van Demark (1945,a)	1:8	-	469	49.3	(a) At 1:50 dilution average spermatozoal content was 26 million per ml. Most inseminations were done with 2 and 3 day old semen but data were insufficient to show any lowering of conception rate with stored semen.
	1:12	-	623	49.9	
	1:16	-	714	46.2	
	1:24	-	796	46.7	
	1:50	-	694	48.7	
			3296	48.0	
Salisbury (1946,b)	1:40	-	1331	54.2	(a) At 1:100 dilution average spermatozoal density was approx. 13 million per ml. Most semen was used on 2nd, 3rd and 4th day after collection.
	1:60	-	1386	52.3	
	1:80	-	1501	54.6	
	1:100	-	1547	56.2	
Rottensten and Andersen (1956)	1:15	-	14596	53.5	(b) The difference in conception rates of fresh and 1 day old semen varied between 3.2 and 2.7 per cent at all dilution levels. Conception rate at 1:15 dilution was significantly greater than these at 1:31 or 1:47 dilution levels.
	1:31	-	14462	51.2	
	1:47	-	14580	50.6	

Ref.	Dilution Rate	Age of semen (1)	Number of inseminations	Conception Rate per cent(2)	Remarks
Stewart (1950)	1:20	2nd day	1409	67.1	(c) Drop in conception rate from 2nd to 4th day was approx. 9 per cent at both dilution levels.
		3rd day	1336	59.7	
		4th day	534	58.4	
			3279	62.7	
	1:50	2nd day	1173	65.7	
		3rd day	1022	63.5	
		4th day	366	56.3	
			2561	62.7	
Melrose (1952,b)	1:50	1st day	678	69.6	(c) Drop in conception rate from 1st to 3rd day was approx. 10 per cent at both dilution levels.
		2nd day	734	65.5	
		3rd day	705	58.7	
			2117	64.6	
	1:100	1st day	752	68.6	
		2nd day	728	62.9	
		3rd day	760	58.8	
			2240	63.4	
Willett (1953)	1:100	1st day	1590	70.1	(d) Conception differences (5.8 per cent) at two dilutions were highly significant P<0.001. There was no significant interaction between age of semen and dilution level; but total differences between 1st and 2nd day semen conception rates (5.9 per cent) was highly significant (P<0.001).
		2nd day	1697	66.5	
			3287	68.2	
	1:300	1st day	1583	66.8	
		2nd day	1737	58.6	
			3320	62.4	

Ref.	Dilution Rate	Age of semen (1)	Number of inseminations	Conception Rate (2) per cent	Remarks
Bratton, Foote and Henderson (1954,a)	Number of motile spermatozoa per insemination				
	14.3 million		4,100 approx.	70.5	(d) Conception rate differences significant ($P<0.05$)
	9.5 million		4,100 approx.	70.9	
	4.7 million		4,100 approx.	66.7	

Note:- (1) 1st day = day of semen collection.
2nd day = day after semen collection.

(2) (a) 5 month non-return conception rates
(b) percentage actually pregnant
(c) 112 day non-return conception rates
(d) 60-90 day non-return conception rates

Salisbury, Elliott and Van Demark (1945,a) and Salisbury (1946,b) demonstrated that with egg yolk-citrate at dilution rates up to 1:100 there was no adverse effect on fertility; no antibiotics or sulphanilamide were used in these trials and the minimum number of spermatozoa used for insemination was approximately 13 million. The later investigation, reported by Melrose (1952,b), was in accord with the foregoing report. In this study, there was no evidence of any effect of dilution up to the 1:100 level on the overall fertility, even when the semen was used for up to 3 days after collection; there was, however, a definite decline in conception rate from the 1st to 3rd day of use of semen at all dilution rates, even when streptomycin was included in the diluent. Bonadonna (1950,a), however, in a study of dilution rates up to 1:20, obtained an improved conception with semen used at the higher dilutions. Following an investigation into dilution over 1:100, Salisbury and Bratton (1948) indicated that the progressive decrease in fertility, which occurred in yolk-citrate, was reduced with the addition of sulphanilamide to the diluent. However, only 356 first and second inseminations were performed with semen used at different dilution rates in yolk-citrate compared with 7,343 first inseminations with semen diluted at varying levels in yolk-citrate plus sulphanilamide; also, since these trials were carried out separately, the two results are not strictly comparable. Although these inseminations were done with semen stored for 2 to 4 days after collection, there is no reference in the report to the possible effect of storage on conception

rates; these authors suggested that the minimum number of spermatozoa required for optimum fertility lay between 5 and 10 million per insemination. Willett (1950), however, reported that the threshold dilution level may vary between bulls; in some this could be as low at 1:80, but in the main the reports confirmed that above the 1:100 level the decline in fertility bore a curvilinear relationship with the number of spermatozoa inseminated. As this number decreased there was an 0.5 per cent reduction in conception rate for each million fewer spermatozoa used per insemination until below the 6 million level, when conception rate declined 2 to 6 per cent per million decrease in spermatozoa. Since these data were obtained with semen used up to 36 hours after collection the findings could not be considered to be applicable for longer storage periods. However, Willett and Larson (1952) attributed the 7.8 per cent difference in conception rate, with semen diluted at the 1:100 and 1:300 levels with and without antibiotics, to the decrease in spermatozoal numbers at the high dilutions and not to the effect of dilution; when the non-return rates for inseminations were adjusted to allow for equal numbers of spermatozoa per insemination they did not differ significantly, in spite of differences in the actual semen dilution rate. The conception rates following the use of 4 million spermatozoa per insemination were 3 per cent lower than those obtained when 12 million spermatozoa were used. In a subsequent report, Willett (1953), using yolk-citrate plus sulphanilamide in 3 experiments involving 6,607 inseminations, found an 8.2

per cent decline in conception rate with semen stored for one day at a dilution of 1:300 compared with a 3.6 per cent decline with semen stored for a similar period at a dilution of 1:100. Although Rottensten (1952) reported significant conception rate differences with 1:8 and 1:16 dilution levels, in a subsequent publication, Rottensten and Andersen (1956), using dilution rates from 1:15 up to 1:47, found that the numbers of spermatozoa inseminated did not affect the conception rate, but the latter was adversely affected by the length of storage of semen.

Again using semen stored in the presence of antibiotics and sulphanilamide for one to two days after collection, Bratton, Foote and Henderson (1954,a) found a relationship between the number of motile spermatozoa per insemination and conception rates, 9.5 million motile spermatozoa giving a conception rate of 70.9 per cent whereas with 4.7 million motile spermatozoa (i.e. at a dilution rate of above 1:300) it was significantly lowered to 66.7 per cent. These authors made no reference to any lowering of fertility with the stored semen. This assessment of spermatozoal requirements was expressed in terms of motile spermatozoa and not as total spermatozoa, and is therefore not in accord with the numbers quoted by Willett (1950) who referred to the total spermatozoan requirements.

Branton, Kellgren and Patrick (1953,a) recommended that dilutions should be made on the basis of a minimum of 6 million motile spermatozoa per insemination to ensure a uniform level of fertility, and Olds, Seath, Carpenter and Lucas (1953) presented evidence of a decline in

fertility when the total number of spermatozoa per insemination was decreased from 12 to 8 million.

The above reports indicate that dilutions up to 1:100 can be made with samples of average quality; however, if higher dilution rates are required 10 million spermatozoa per insemination should be retained. The motility rating of the particular sample should be taken into account since at least 5 million motile spermatozoa appear to be required. In general, these recommendations have been based on results obtained with semen used up to 36 hours after collection and may not apply with semen stored for longer periods.

B. Age of Semen

An extensive study of the effect of this on conception rates with 25,146 inseminations over a two year period was reported by Schultze, Davis, Blunn and Oloufa (1948), who found the average conception rate reduction per day of storage up to 4 days to be 4.6 per cent; these results were obtained with a yolk-phosphate diluent with dilution rates up to 1:30. Subsequently, Campbell (1953), from observations based on 50,213 inseminations carried out from 2 insemination centres over 4 six monthly periods, showed the decline in the average conception rate for each day of storage up to 4 days to vary between 3.4 per cent and 8.3 per cent, according to the centre and the 6 month period studied. Similar findings have been published by Aamdal (1952), Dzilinski (1958) and Fryer, Marion and Farmer (1958).

Although it is well established that the highest fertility results with semen used on the day of collection, there is also evidence that

the decline in fertility is often of a low order (i.e.up to 3 per cent) within 30 hours after collection (Melrose, 1952,b; Willett, 1953; Hewetson, 1955; Rottensten and Andersen, 1956); however, Roussel (1954) in a study with 36,000 cows found there was a 5 per cent drop in conception rate for every 24 hours of semen storage.

The rate of decline in fertility was claimed by Stewart (1950) to be associated with the semen quality and fertility level of the individual bull. However, the diluent may also influence this; for example, with milk diluent the 1st and 2nd day conception rate differences were normal but the decline on the 3rd day of storage was in excess of that with yolk-citrate (Perkins, Carpenter and Seath, 1955). The reduction in fertility after storage is of economic importance in commercial artificial insemination, and the use of diluted semen stored at refrigerator temperatures for longer than 48 hours has not been extensively practised. However, the development of diluents to extend the period of storage appears to show promise (see page 113) and could be of practical importance both in isolated districts and in the provision of nominated services from proven bulls. There is little doubt, however, that the use of stored semen requires much stricter attention to the technique of handling and dilution than is required with semen used on the day of collection or at low dilution rates.

C. Cooling of Semen

This is aimed at prolonging the fertilisable life of the spermatozoa by reducing the metabolic reactions in the semen to a low level. Anderson and Seath (1948) showed that spermatozoal survival was improved by diluting as soon as possible after collection of the semen. Strict attention

must be paid to the prevention of damage to the spermatozoa by "temperature shock", as demonstrated originally by Chang and Walton (1940). Temperature shock has been further studied by Mann and Lutwak-Mann (1955) and by Blackshaw and Salisbury (1957), who showed that it increased the permeability of the cell wall with the resultant outflowing of essential elements. Even although Phillips and Lardy (1940) showed that the use of an egg yolk buffer greatly minimised this risk, the cooling of semen diluted 1:4 in yolk-phosphate or yolk-citrate diluents at the very rapid rate of 30°C. per minute was shown by Willett and Salisbury (1942) to adversely affect motility. However, Foote and Bratton (1949), in a split sample study involving cooling from 30° to 5°C. over 75 minutes, found that semen, diluted 1:4 in yolk-citrate plus sulphanilamide, cooled to 5°C. and thereafter further diluted, had a significantly higher conception rate and motility rating than the semen which was cooled undiluted, but at a similar rate, to 5°C. and then diluted to the same extent. The conception rate differences could not be accounted for by variations in the estimated numbers of motile spermatozoa. In both cases the degree of final dilution, based on the pre-cooling motility, allowed 10 to 25 million motile spermatozoa per ml., but variations in the post-cooling motility ratings with the different treatments resulted in differences in the estimated number of motile spermatozoa used for insemination. In this study, it was claimed that the higher conception rate could have been due to the protective action of the egg yolk or to the partial dilution of the raw semen prior to cooling or to both these procedures.

Laing (1955) recommended the holding of the freshly collected semen in a water bath at 20°C. pending its examination and mixing with the diluent (also at 20°C.). Presumably this was based on the report of Chang and Walton (1940) that the risk of temperature shock in cooling semen increases as the temperature is lowered and markedly so below 20°C. However, many workers prefer to do the initial dilution at 30°C., as recommended by Anderson (1945), but this increases the time required to cool the diluted semen to 5°C. Aehnelt (1952), using an egg yolk-Spermasol diluent, claimed that dilution at a temperature of 20°C. gave optimal spermatozoal survival rates, but he also found that a secondary dilution after storage at 5°C. for 24 hours adversely affected motility.

Although there is only a limited amount of fertility data on the effect of diluents in relation to cooling rates, the practice of diluting or at least partially diluting the semen prior to cooling has been almost universally adopted.

D. Effect of transport

Attention has been drawn by Prince and Almquist (1948), and subsequently by Van Demark, Salisbury and Bratton (1949), to the detrimental effect on motility of storing semen in partly filled tubes. This was presumed to be due to increased availability of oxygen to the spermatozoa, especially when the tubes are shaken in transit. Although Letard, Szumowski and Arruti (1949) confirmed this in their study of the effect of agitation of undiluted semen in a partly filled tube in the presence of air, when diluted semen was used

the motility was increased by agitation for 4.5 hours; however, the survival rates of the agitated semen after storage were not given. Salisbury and Sharma (1957) showed by manometric measurements that at 37°C. the oxygen uptake of spermatozoa varied according to the exposed surface area of the diluting fluid, which in this case was phosphate buffer or normal saline without egg yolk. The transport of the diluted semen in single insemination doses in paraffin coated straws, as devised by Sorensen (1946), or in single dose glass tubes, as described by Ferreira-Barreto and Mies-Filho (1949), would appear to overcome this problem; this would, however, entail extra work and also extra handling of the semen in order to fill the tubes. This has not been assessed in terms of its effect on fertility and there has been no general use of single dose containers for fresh semen with conventional diluents except in Norway where Aamdal (1955,a) has reported on the extensive routine use of single dose polyethylene tubes closed by a bark cork. Plastic containers, which can be discarded after use, present fewer difficulties in packing and cleansing than do glass containers; the report by Dunn and Welker (1957), who found no differences in conception rate between semen transported in glass or plastic containers, indicated that plastic containers could be safely used; this investigation covered over 23,000 first inseminations.

E. Heterospermic inseminations

Kusner (1954), in a review, indicated that the best conception rate could be obtained with a mixture of semen from 3 or 4 breeds. Frappell and Williams (1956) were unable to demonstrate any beneficial

effect on fertility by the use of mixed samples of semen from 10 Hereford bulls; the observation that there was a high conception rate at one bull stud with mixed Hereford semen (74-82 hours old) was, however, uncontrolled and only on a limited number of inseminations. Hess, Ludwick, Richard and Ely (1958) in a controlled trial involving 1,422 first inseminations reported a significantly higher conception rate with mixed semen prepared from 51 ejaculations from 47 different bulls, a portion of the ejaculates from 3 or 4 different bulls being mixed together on each collection day. Metabolism studies on mixed semen samples carried out by Ehlers, Kushwaha and Erb (1958) revealed an increased fructolysis and consequent increased lactic acid accumulation; part of this rise in fructolysis may have been due to the increased fructose content of some samples after mixing. Dott and Walton (1958) could find no effect on the ratio of live : dead spermatozoa or on the percentage of abnormal forms following the mixing of semen from different bulls; the motility rating of the mixed samples was nearer to that of the least active than to the mean of all the samples. In the motility studies carried out by Campbell and Jaffe (1958), the motility rating for the mixed sample was lower than that of the better sample included in the mixture. No clumping effect following the mixing of the semen was reported. From this evidence it would appear that further investigation is required into the possible beneficial effect of heterospermic insemination.

F. Handling of diluted semen in the field

Although there is a lack of published evidence on the influence

of this on conception rates; in particular when stored semen is being used the methods adopted must ensure that the laboratory procedures for prolonging the fertilising capacity of the spermatozoan can also be adopted in the field. The deleterious effect on spermatozoal motility and livability of allowing the diluted semen to be shaken about in half filled test tubes was demonstrated by Van Demark et al. (1949) who indicated this could be due to the increased oxygenation of the spermatozoan. The risks of a lowered conception rate by packing the semen in thermos flasks in such a manner as to reduce the cooling effect of the ice, and also the need to avoid the carrying of the same tubes of diluted semen by operators on successive days, were referred to by Melrose (1952,b), who reported a 10 per cent improvement in the conception rate, following attention to these factors; however, since the fertility results for a period of 6 weeks before and 6 weeks after attending to these aspects of the techniques were compared this author's findings were not based on a properly controlled trial.

S E C T I O N VIII

ANTIBIOTICS IN SEMEN DILUENTS

Bacteria in semen - methods of control, use of antibiotics etc.

The risk of transmitting pathogenic bacteria in semen was apparently appreciated by Iwanow (1917), who investigated the addition of various chemical compounds to the semen; however, since these compounds were also spermicidal, no benefit was gained from these additives. Salisbury, Willett and Gunsalus (1939) drew attention again to the need for the control of bacterial contamination in view of its possible effect on semen storage; the advent of the egg yolk diluent, which permitted the use of semen stored for longer periods than hitherto, also provided a suitable medium for bacterial survival and growth; this resulted in the appearance of many publications on the bacterial contamination of semen and also on the use of sulphanilamides and antibiotics in semen diluents.

A. Bacteria in semen and their possible significance

Although strict attention to hygiene at the time of collection can keep bacterial contamination down to a minimum, there appears little doubt that semen samples completely devoid of bacteria are almost impossible to obtain. Rozsa (1950) reported that semen, collected regularly from 8 healthy bulls, contained an average of 80,000 bacteria per ml. (range 250 to 400,000) and that a combination of penicillin and streptomycin could effectively control the growth of these bacteria, even when the semen was stored for up to 12 days

in yolk-citrate diluent at 4°C. The inhibitory action on bacterial growth of penicillin and streptomycin in diluted semen was confirmed by Alford (1953); however, in thisstudy bacterial plate counts of over 10,000 per ml. were also obtained even in the presence of sulphanilamide, penicillin and streptomycin, but there was no evidence of any adverse effect of these on fertility. In addition, there is the risk of bacterial contamination being introduced into the diluent at the time of its preparation. The reports on the possible effect of bacterial contamination on fertility have been conflicting. Gunsalus, Salisbury and Willett (1941) claimed that Pseudomonas aeruginosa in semen could adversely affect its fertility and later Bush, Ludwick, Ferguson and Ely (1950) demonstrated a correlation between fertility and the bacterial count of the diluted semen, the chief bacterial species responsible for this being Corynebacterium pyogenes, pseudomonads and micrococci. However, Almquist, Prince and Reid (1949) were unable to demonstrate any relationship between either the number or types of bacteria present in semen and its fertility, Pseudomonas aeruginosa having been found in semen of both high and low fertility bulls.

These studies were principally concerned with those bacteria, normally regarded as contaminants. There is ample proof that known pathogenic bacteria may be present in semen and spread disease by this vehicle. The spread of Brucella abortus in infected semen used for insemination has been reported from Denmark by Seit (1944) and Bendixen and Blom (1947). In the course of investigations ~~into~~

into abortions due to Vibrio fetus infection, Plastridge, Williams and Petrie (1947) drew attention to the lowered conception rates occurring in herds in which positive serum titres against V.fetus had been found in both cows and bulls, indicating that the latter could be a possible source of spread of the infection. An enzootic sterility spread by insemination, and later shown to be caused by Vibrio fetus, was reported from Holland by Sjollema, Stegenga and Terpstra (1952). Similar findings were reported by Terpstra and Eisma (1951), Flatla Braend and Sudboe (1952) and Lawson and Mackinnon (1952). Although Trichomonas foetus is not commonly found in countries using artificial insemination extensively the risk of spread of this, by insemination from an infected bull, was emphasised by Joyner and Miller (1952). Of the other definitely known pathogenic organisms likely to be present in bull semen, particular attention has been directed towards Leptospira, which were reported to be associated with abortion by Te Punga and Bishop (1953) in New Zealand and Fennestad and Berg-Petersen (1958) in Denmark. Although transmission of this infection by semen has not been proven, Bryan (1955) reported outbreaks of Leptospirosis in herds using inseminations. The significance of the pleuropneumonia like organisms (PPLO), demonstrated by Edward, Hancock and Mignett (1947) in bull semen and in the genital tract of infertile cows, is not at all clear; Albertsen (1955) found that 89 per cent of 85 semen samples, examined at two Danish insemination Centres, were carrying these organisms. Bakos, Bane and Thal

(1959) found PPLO in the semen of 10 out of 20 fertile, and in 14 out of 25 low fertility bulls, and considered they were not a primary cause of genital disease in cattle; various types of these organisms exist and further study on them is required.

Corynebacterium pyogenes is commonly found in bull semen (Hancock and Kelly, 1948), and must, presumably, be frequently spread by insemination; the circumstances under which this organism becomes associated with impaired fertility have not been clearly demonstrated.

Whilst there can be no distinct differentiation between the pathogenic and commensal or contaminant bacteria, the proved pathogenic bacteria do not multiply greatly, if at all, under the conditions in which the semen is stored, while many of the so-called contaminants do and may become important because of the resultant putrefaction; for example, Ps. aeruginosa (Foote and Salisbury, 1948,b) - appears to be able to multiply in semen stored at 5°C. (and in particular in the presence of egg yolk) with resultant putrefaction of the semen.

B. Addition of anti-bacterial substances to semen diluents

When chemotherapeutic agents and antibiotics became readily available, the effect on conception rate of their addition to semen was determined, although there was, at that time, no exact information on the importance of bacterial contamination in semen. A summary of certain of these studies is given in table 9.

(i) Chemotherapeutic agents. Salisbury and Knodt (1947), Foote and Salisbury (1948,b), Salisbury and Bratton (1948), Bratton, Foote,

TABLE 9

SUMMARY OF STUDIES WITH ANTIBACTERIAL AGENTS IN SEMEN DILUENTS

Ref.	Antibacterial Agent	Number of inseminations	Conception rate per cent	Remarks
Salisbury and Knodt (1947)	(I)			
	none	786	48	(a) Diluent not protected from sunlight.
	sulphanilamide	767	48	
	(II)			
	none	676	56.1	(a) Direct light excluded from diluted semen. Authors suggested sulphanilamide had a metabolic and not an anti-bacterial effect.
	sulphanilamide	765	62.2	
	(III)			
	none	2484	59.7	
	sulphanilamide	2300	64.2	
Foote and Bratton (1950,b)	none	933	61	(b) Conception rate differences not significant, but low fertility bulls showed on the average 10 per cent higher conception with the antibiotic addition.
	sulphanilamide	927	62	
	penicillin	885	68	
	streptomycin	907	68	
	polymyXin	862	64	
	combination of above	845	68	
Stewart (1950)	none	1786	61.5	(c)
	sulphanilamide	1583	62.3	

Ref.	Antibacterial Agent	Number of inseminations	Conception rate per cent	Remarks
Holt (1952,b)	none	4428	69.8	(d) No difference recorded in drop from 21 day to 3 month non-return conception rates with either treatment.
	sulphanilamide	4304	70.9	
Easterbrooks, Heller, Plastridge, Jungherr and Elliott (1950,b)	sulphanilamide	2340	61.6	(b) Conception rate difference highly significant.
	sulphanilamide plus streptomycin	2379	69.8	
Adler, Lange and Rasbech (1952)	sulphanilamide	8166	59.7	(b) Significant conception rate increase confined to certain bulls.
	sulphanilamide plus streptomycin	8095	62.2	
Almquist (1951,b)	(I)			(e) Investigations done with 7 relatively infertile bulls.
	none	486	35 *	
	penicillin	475	54	
	(II)			* Differences in conception rate highly significant. ($P<0.01$).
	none	540	34 *	
	streptomycin	523	60	
	(III)			
	none	525	36	
	penicillin and streptomycin	519	59 *	

cont.....

Ref.	Antibacterial Agent	Number of inseminations	Conception rate per cent	Remarks
Almquist (1951,b) cont...	(IV) none	420	37	
	sulphanilamide	430	33	
Stewart, Melrose and Wilson (1951)	none	5394	56.6	(c) Significant conception rate increase confined to certain bulls of low fertility in diluent without antibiotics.
	streptomycin	5494	66.1	
Hendrikse and Joling (1952)	sulphanilamide	1283	55.5	(f)
	sulphanilamide plus penicillin and streptomycin	879	56.9	
Aamdal (1955,b)	none	2737	57.3	(g) No significant difference between treatment with any of the 18 bulls used.
	penicillin plus streptomycin	3284	56.5	
Adler and Rasbech (1954)	(I) sulphanilamide	5058	57.5	(a) Conception rate increased with V. fetus infected bulls.
	sulphanilamide plus neomycin	5617	58.4	
	(II) sulphanilamide plus streptomycin	8329	65	
	sulphanilamide plus neomycin	8901	62.6	

Ref.	Antibacterial Agent	Number of inseminations	Conception rate per cent		Remarks
Willett and Ohms (1955)	none	7570	53.9	(b)	Results obtained with 23 bulls (some V. fetus infected) showing a significant increase in conception rate with the sulphanilamide and streptomycin addition.
	sulphanilamide and streptomycin	7578	70.6		
	streptomycin	7651	68.1		
	penicillin and streptomycin	7729	67.0		
	none	16683	68.0		Result obtained with 67 bulls showing no conception rate increases with antibiotic addition.
	sulphanilamide and streptomycin	16459	70.6		
	streptomycin	16445	68.6		
	penicillin and streptomycin	16450	67.7		
Adler and Rasbech (1956,a)	streptomycin	11391	65.7	(a)	
	streptomycin plus sulphanilamide	11368	64.3		

Note:- conception rates assessed at following intervals after insemination:

(a) 5 months
(b) 60-90 days
(c) 112 days
(d) 90-120 days
(e) 6 months
(f) at calving (g) at pregnancy diagnosis.

Musgrave and Van Demark (1949) and Karlshoj and Rasbech (1949) found that when sulphanilamide (usually 300 mg./100 ml.) was added to diluted semen, an increase in spermatozoal survival and also an increased conception rate resulted. Whether or not these increases were due to antibacterial action alone seems doubtful. Knodt and Salisbury (1946) showed that the addition to semen of sufficient sulphanilamide to keep bacterial multiplication at a low level over 72 hours (200 mg./100 ml. semen) also caused a reduction in the oxygen uptake of the spermatozoa and a stimulation of glycolysis; also this agent was claimed to have a beneficial effect with all bulls. However, Stewart (1950) and Holt (1952,b) were unable to confirm that sulphanilamide increased the conception rate and Adler and Rasbech (1956,a) reported a slight lowering of the conception rate with this. Van Demark, Bratton and Foote (1950) and Foote and Bratton (1950,b) found that the differences in conception rates with yolk-citrate and yolk-citrate plus sulphanilamide diluents were only about 1 per cent as compared with the 5 per cent increase reported previously by Salisbury and Knodt (1947). These authors pointed out that the absence of the beneficial effect of the sulphanilamide in the recent work was due to changes in the method of cooling the diluted semen subsequent to 1947.

Foote and Salisbury (1948,b) made a study of the effect of 12 other sulphonamides on spermatozoal survival in yolk-citrate and found that only sodium sulphamezathine and carboxylsulphathiozole were superior to sulphanilamide in prolonging spermatozoal motility.

Since these agents were not so effective in controlling bacterial growth, their use in semen diluents was not adopted. In a similar investigation, Foote and Salisbury (1948,a) found that pyridium, furacin and phenoxethol were toxic to spermatozoa at the required bacteriostatic concentration.

(ii) Antibiotics. An improved conception rate has been shown to result from the addition to semen of either penicillin (Almquist, 1948; Foote and Bratton, 1950,b) or of streptomycin (Easterbrooks, Heller, Plastridge, Jungherr and Elliott, 1950,b; Foote and Bratton, 1950,b; Almquist, 1951,b; and Stewart, Melrose and Wilson, 1951), and these have generally been used in preference to other antibiotics which are more toxic to spermatozoa. However, Van Dieten (1953), Hendrikse and Joling (1952) and Aamdal (1955,b) observed no overall effect on fertility following the inclusion of penicillin and streptomycin in the semen diluent.

In these reports 500 - 1000 I.U. of penicillin and 500 - 1000 µg/ml. streptomycin were used. Although considerable attention has been paid to the bactericidal effect of the clinical dose levels, there are very few reports on the bactericidal effect of the high concentrations commonly used in semen diluents; Albertsen (1957) pointed out the danger of concluding by analogy that the results, obtained with the low concentrations, can also be applied to the high concentrations. For example, there is evidence in the literature that the addition of streptomycin calcium chloride at a level of 500 µg/ml. of diluent is equally as effective, as assessed by the resultant

conception rates, as 1000 μg/ml. (Easterbrooks, 1951; Melrose, 1953; Rottensten, 1954).

(a) Penicillin. Although the risks of toxic effects with this are very slight, Foote and Salisbury (1948,a) reported that occasional samples may be toxic. Using spermatozoal survival tests, a level of 5,000 units per ml. of diluent was found to be satisfactory by Hennaux, Dimitropoulos and Cordiez (1948), Knodt and Salisbury (1946), Sykes and Mixner (1951), and White (1954,b), but Myers and Almquist (1951) found, in their investigation, that 1000 units/ml. were toxic after storage for 20 days at 5°C. Since the beneficial effect on conception rates was obtained with levels up to 1000 units/ml. diluent, there would appear to be little merit in exceeding this level.

(b) Streptomycin. The work of Myers and Almquist (1951) showed that spermatozoa could tolerate streptomycin at levels up to 1000 μg/ml. diluent. Hennaux, Dimitropoulos and Cordiez (1947) demonstrated an improvement in motility with levels as low at 300 μg/ml. Almquist (1951,b) obtained a 27 per cent increase in conception rate with relatively infertile bulls when 1000 μg streptomycin was added per ml. of semen diluent, but Easterbrooks, Heller, Lieberman, Plastridge and Jungherr (1951) and Easterbrooks et al. (1950,b), using only one tenth of this dosage, i.e. 100 μg/ml., increased the conception rate from 61.6 per cent to 69.8 per cent; these authors also advocated the use of 500 μg/ml.,

the dosage required to kill V. fetus. Rottensten (1954) confirmed that 500 µg/ml. diluent was as effective as the 1000 µg level, whereas Adler and Rasbech (1953), using V. fetus infected bulls, reported a high conception rate with dihydrostreptomycin sulphate used at 5000 µg/ml. compared with 1000 µg/ml. For practical working, which would allow for a margin of safety under field conditions, a safe level would be 750 µg/ml.

Whilst most of the original workers used the streptomycin calcium chloride complex, at present dihydrostreptomycin is often used. Easterbrooks, Heller, Plastridge and Jungherr (1950,a) found no significant effect on fertility when dihydrostreptomycin sulphate was used instead of streptomycin calcium chloride, and Albertsen (1957) found these two streptomycin salts to have an equal bactericidal action when using Br. abortus as a test organism. At the Reading Cattle Breeding Centre (Report, 1959,b) no difference in fertility was demonstrated when these two types of streptomycin were compared in over 2,217 inseminations. However, Easterbrooks et al. (1950,a) pointed out that the calcium chloride complex cannot be used with phosphate buffers as, if this streptomycin salt is used at concentrations of over 100 µg/ml. in the buffer, it will be precipitated. A reduction in conception rate, presumed to be due to this effect, was reported by Campbell and Edwards (1955). There is, therefore, no reason why the now readily available and equally effective dihydrostreptomycin should not be used.

(c) Oxytetracycline. At a level of 500 µg/ml. this was found by Albertsen (1957) to have a bactericidal effect in yolk diluent only slightly less than that of streptomycin. As this level would appear

to be toxic to spermatozoa, 200 μg/ml. having been previously given as the safe level by Stallup and McCartney (1953), the use of oxytetracycline in the diluent could not be recommended.

(d) Chlortetracycline. Foote and Bratton (1950,a) reported that a level of 100 μg/ml. in yolk diluent would control bacterial growth and not adversely affect spermatozoal survival but Myers and Almquist (1951) claimed that levels over 50 μg/ml. were spermicidal. Although these findings were based on storage test results, which were perhaps not strictly comparable, it would appear that over 50 μg/ml. could be toxic. Easterbrooks (1951) claimed a 4 per cent higher conception rate with the addition of 50 μg/ml. when compared with streptomycin (500 μg/ml.), but no other extensive fertility trials of this have been carried out. Albertsen (1957) found that, in the presence of egg yolk, 50 μg/ml. had little or no effect on the Br. abortus, V. fetus or C. pyogenes. In view of its doubtful effect in egg yolk at the levels which have the least spermicidal action, there is no clear indication for the use of this in preference to , for example, streptomycin.

(e) Chloromycetin. Here again there is little information on the usefulness of this in diluents; Easterbrooks (1951) obtained an improved conception rate (3 per cent) when the addition of 500 μg/ml. of chloromycetin was compared with the usual streptomycin addition, but Albertsen (1957) reported that 1000 μg/ml. had little or no bactericidal effect in egg yolk diluent. Little published data is available indicating any benefit with this over streptomycin.

(f) Neomycin. Motility studies by Di Liello, Poelma and Faber (1957) indicated that a level of 500 µg/ml. would not be spermicidal, but Albertsen (1957) found that at 100 µg/ml. in yolk diluent no control of growth of the pathogenic bacteria studied could be expected. This was also confirmed in a controlled field fertility trial by Adler and Rasbech (1954) who showed that 500 µg/ml. of neomycin did not increase the conception rates of the low fertility bulls to the same extent as occurred with the streptomycin addition. The conception rates were 62.6 per cent and 65 per cent with neomycin and streptomycin, respectively, in a trial involving 17,200 inseminations.

(g) Polymyxin. Foote and Bratton (1950,a) claimed that 2000 µg/ml. Polymyxin, D, B, and E, had a high bactericidal action in a yolk-citrate diluent without any toxic effect on spermatozoa. Although a beneficial effect on fertility resulted when these authors used it in combination with other antibiotics, no improvement with its use alone has been recorded.

In general, therefore, the effects on fertility of the addition of the wide spectrum antibiotics to semen diluents have not been extensively investigated, but the results available do not indicate any material benefit could be expected over that obtained with, for example, streptomycin.

(iii) Antibiotic combinations. Although there are many published reports on studies of the effect of these on the bacterial content of semen, on spermatozoal survival and also on fertility, the

results, which have not always been obtained under strictly comparable conditions, have been assessed by different criteria, and are often conflicting. The benefits to be gained from using a combination of antibacterial agents, as opposed to a single antibiotic, have not always been clearly demonstrated and there is also the practical disadvantage of the extra work involved when several antibiotics have to be added to the diluent.

Published reports on the effect of sulphanilamide, penicillin and streptomycin, used either singly or in combination, are contradictory but, as far as the antibiotics are concerned, the chances of a resultant improved conception rate with their addition would appear to depend on the fertility level of the bulls used in the trial. Almquist, Thorp and Knodt (1948); Olds, Oliver and Seath (1951,a); Mixner (1949, a and b); Hendrikse and Joling (1952); Van Dieten (1953) and Aamdal (1955,b) got no increased conception rate with the addition of penicillin and streptomycin but this was possibly because there was no infection in the bulls used. Adler, Lange and Rasbech (1952) and Adler and Rasbech (1952) reported the greatest increase in conception rates with bulls of low fertility with the addition to the sulphanilamide yolk-citrate of streptomycin (up to 5000 µg/ml). Also, Rottensten (1953) obtained the greatest conception rate increases with streptomycin alone but Willett and Ohms (1955) got the maximum increase in fertility when using streptomycin and sulphanilamide together. Erb, Mikota, Flerchinger and Ehlers (1955,b), found that 1000 µg/ml. of streptomycin gave the best response whereas with 500

µg/ml. of streptomycin, alone or in combination with penicillin, the increase was slightly less. The antibiotic effect on low fertility bulls was confirmed by Sakala (1957) who reported conception rates of 54.8 per cent and 48.5 per cent with streptomycin and penicillin treated and untreated semen respectively; although this was a split-sample study, involving 7,533 cows, there was an abnormally large disparity in the numbers of inseminations in each treatment group (2,923 compared with 4,610).

An extensive field trial, involving 69,000 first inseminations for which sulphanilamide, streptomycin and penicillin were used singly, or in combination, in yolk-citrate or in yolk-phosphate buffer, was reported by Campbell and Edwards (1955); for various reasons 10 per cent of the recorded inseminations were not included in the analysis of the data. With the citrate-yolk diluent, the combined addition of sulphanilamide, streptomycin and penicillin improved the conception rate by 6.8 per cent and with streptomycin alone the increase was 5.4 per cent; the difference between these increases was not significant. However, with yolk-phosphate, penicillin alone gave a conception rate 9.3 per cent above that with yolk-citrate alone, and this was not significantly greater than that with yolk-citrate plus the antibiotic combinations. Sulphanilamide and streptomycin, alone or in combination with each other or with penicillin, in yolk-phosphate gave a lowered conception rate, which was attributed to the incompatability (see page 169) of the streptomycin calcium chloride and the phosphate buffer. Although the highest conception rates in the trial were obtained with yolk-phosphate

plus penicillin, the authors did not recommend its general adoption since it did not give a clear field for semen examination. In view of this, further investigations into the use of phosphate buffers with antibiotics would appear to be advisable.

In general streptomycin appears to be the most active antibiotic; it has been shown to be the most effective against V. fetus but whether or not it should be used singly or in combination with sulphanilamide and penicillin has not been definitely established.

(iv) Mode of action of antibiotics. Whilst the above reports did establish that an increased conception rate could be obtained, the exact reason for this effect was not demonstrated. Although many authors implied that the reason was an anti-bacterial action, no supporting bacteriological investigations were carried out to prove this in spite of the fact that most workers showed that the increase in conception rate was confined to certain bulls of low fertility. Furthermore, Foote and Bratton (1952), Erb et al. (1955,b) Willett and Ohms (1955) and Melrose (1953) pointed out that at least part of this increase in fertility possibly resulted from a reduction in embryonic mortality when the semen from these low fertility bulls was treated with antibiotics, as indicated by the reduction in the difference between the 30-60 day and 90-120 day non-return conception rates.

Although it was originally intended to inhibit bacterial multiplication in stored semen, it was eventually shown that in a many cases the improved conception rate was due to the killing off of certain pathogenic bacteria, which, although incapable of multiplication

under the conditions in which semen was stored, were being spread in this vehicle. Chief among these was V.fetus. Albertsen (1957) pointed out that with saprophytic bacteria the establishment of merely bacteriostatic conditions was satisfactory while with pathogenic organisms present the antibiotics had to be bactericidal to be effective. When this was realised, experiments were carried out to determine the conditions under which various antibiotics showed their maximum bactericidal action against pathogens and also which antibiotics were the most efficient. Albertsen (1957), using C. pyogenes, Br. abortus and V.fetus as test organisms in the yolk-citrate diluent, found that various antibiotics including penicillin, oxytetracycline, streptomycin, chlortetracycline, chloromycetin, had no effect upon these bacteria after 24 hours' storage at 5°C., but ~~that~~ at higher temperatures ~~of~~ (15°C. to 30°C.) bacterial destruction was increased, although the different antibiotics acted differently. Streptomycin was the most effective. Oxytetracycline, although definitely bactericidal, was less active. However, penicillin, neomycin and chlortetracycline were completely ineffective against the organisms tested and the author concluded that these must be considered to be quite unsuitable for addition to semen for the purpose of destroying pathogenic bacteria. This work has been confirmed by Morgan (1958), and whilst these results are in agreement with those previously reported by Plastridge and Easterbrook (1952) and Lenz (1956), they are contrary to those of Orthey and Gilman (1954), Hughes (1956) and Adler (1957), who claimed that V. fetus could be effectively controlled by the use of antibiotics

under similar storage conditions.

In order to ensure the maximum bactericidal effect with antibiotics Albertsen (1957) recommended that prior to cooling the diluted semen should be stored at 37°C. for 15 minutes, or at 30°C. for 1 hour, as this would ensure almost complete destruction of e.g. V. fetus and C. pyogenes. However, Lawson (1954) was still able to infect heifers with V. fetus infected semen, which, after dilution in the egg yolk-citrate antibiotic mixture, had been allowed to stand at room temperature for two hours before storage at 4°C. prior to insemination. Admittedly the infected semen in this experiment was used at a very low dilution rate, but this report, together with the results reported above, indicated that, at least with the yolk-citrate diluent, the antibiotic alone could not always be relied upon to prevent the possible spread of the infection in the semen. No such detailed studies have been made with other pathogenic bacteria. In a limited study, Albertsen (1957) showed that semen diluted 1:10 in a citrate sulphanilamide diluent without egg yolk could protect cultures of C. pyogenes against streptomycin, but with semen diluted 1:100 the bactericidal effect of the antibiotic was increased markedly. This possible protective action of the semen has not been reported on elsewhere, and it would appear to require further study in view of the conflicting reports on the bactericidal effect of antibiotics in diluted semen.

A depressing effect of antibacterial agents on spermatozoal metabolism was claimed by Branton and Prather (1954) to account for the improved in vitro survival of spermatozoa in the presence of penicillin and

streptomycin. Although other workers have reported this improved survival with antibiotics, the possible metabolic effect of these has not been extensively studied.

(v) Effect of diluent on action of antibiotic. The work of Albertsen (1952) suggested that egg yolk in the diluent, by its protective action on the bacteria, was interfering with the action of the antibiotic. However, the fact that Morgan, Melrose and Stewart (1959) found that streptomycin was bactericidal in yolk-citrate at 37°C. would suggest that either this is not the full explanation or that this protective action occurs only at 5°C. During a series of investigations into milk diluents Dreher and Webb (1953) and Melrose, Stewart and Bruce (1958) demonstrated that with certain bulls there was an increased conception rate with the milk diluent over that obtained with the yolk-citrate diluent; antibiotics were used in both diluents. Morgan et al. (1959) also showed that V. fetus infected semen, diluted in yolk-citrate diluent containing 500 µg/streptomycin/ml. and stored for two hours at 5°C., could still infect test heifers, whereas, in a similar investigation using a skim milk powder diluent also containing 500 µg/ml. streptomycin, this infection could not be transmitted to test heifers. In these trials the semen was used at dilution rates of 1:50 and 1:5 and the effectiveness of the antibiotic appeared to be independent of the semen dilution rate.

(vi) Site of deposition of semen in genital tract. Little information on this is available, but Albertsen (1957) reported that, although conditions in the tract may vary from one insemination to

another, the main factors affecting the antibiotic action were (a) the degree of dilution in the oestrous mucus, and (b) the site of semen deposition since the absorption of the antibiotic is much slower in the cervix than in either the uterus or the vagina. To permit the maximum effect with the antibiotic Albertsen (1957) recommended the intra-cervical site of semen deposition in order to reduce the risk of survival of pathogenic bacteria. The work of Rowson, Lamming and Fry (1953) showed that accidental insemination of cows, in the luteal phase of the oestrous cycle, could result in varying degrees of metritis, and this again could be used as an argument in favour of the intra-cervical site of insemination in order to permit of the maximum protective action by the antibiotic in such cases.

In the early studies anti-bacterial substances were added to semen diluents in an attempt to control bacterial growth and eliminate any risk of spread of pathogenic bacteria. However, in the subsequent work most emphasis was placed on the control of pathogenic bacteria, such as V. fetus, and it would appear that this could account for the improved conception rates reported after the inclusion of penicillin and streptomycin in the diluent. However, further studies of the levels of the appropriate antibiotics required for the control of contaminant bacteria under semen storage conditions would appear to be still necessary. This has become of increased importance in view of the need for prolonging the possible length of storage of diluted semen.

S E C T I O N IX

PRESERVATION OF SEMEN AT ULTRA-LOW TEMPERATURES; HANDLING, DILUTION, GLYCEROLISATION, AND FREEZING TECHNIQUES; FIELD USE OF DEEP FROZEN SEMEN;

A. Introduction and early investigations

Although ultra-low temperature storage has been the most important development in the preservation of semen, Spallanzani, as long ago as the 18th century, had made observations on the effect of snow on stallion spermatozoa (Mann, 1954). The development of means of attaining ultra-low temperatures led to more intensive investigations into the storage of cells and tissues under these conditions. Luyet and Gehenio (1940) postulated that if living cells could be rapidly cooled below zero into what was called the "vitrification zone", the metabolic process of the cell would be so reduced as to enable it to be stored for an indefinite period. Other investigations reported around this time into the effects of low temperature on living cells have been reviewed by Parkes (1956). This author pointed out that, whilst most of the cells and tissues originally studied were adversely affected when subjected to sub-zero temperature, human spermatozoa appeared to be the exception, since they were remarkably resistant to low temperature.

Particular attention was paid by the earlier workers to the rate of freezing, and to the dehydration of the cells or tissues by the use of a concentrated sugar solution. Although Luyet and Hartung (1941) had found ethylene glycol useful as a dehydrating agent in the

freezing of eel worms, and later Rostand (1946) had reported that the addition of 10 per cent glycerol protected frog spermatozoa against the effects of storage at temperatures of -4° to -6°C., it was the chance discovery of Polge, Smith and Parkes (1949) that resulted in these workers investigating fully the possible protective action of glycerol in the deep freezing of, firstly, fowl spermatozoa. Subsequently, work with bull spermatozoa was reported by Smith and Polge (1950). These workers obtained the best survival rates by diluting the semen in a 3.9 per cent sodium citrate buffer containing 15 per cent glycerol and then slowly cooling ampoules of diluted semen to -79°C. This was achieved by passing the ampoules at intervals of 2.5 minutes through 14 progressively colder alcohol baths, previously cooled, by the addition of solid carbon dioxide, to temperatures ranging from 0°C. to -79°C. Stewart (1951) obtained only one pregnancy from five inseminations with thawed out semen that had been deep frozen by almost the same technique; egg yolk and 10 per cent glycerol were used in the diluent. In a more extensive field trial of this technique, Polge and Rowson (1952,a) obtained a nil conception rate. In a second trial a different technique was used. Here the semen was diluted in an equal volume of yolk-citrate buffer, cooled to 5°C. and then further diluted with an equal volume of glycerol-citrate to give a final concentration of 10 per cent glycerol. This diluted semen was left over-night in the refrigerator, ~~and~~ then deep frozen and stored at -79°C. for up to 8 days. A first insemination pregnancy rate of 79 per cent resulted in 38 cows inseminated with it.

In this experiment the diluted semen was reported to have been cooled slowly over a period of 45 minutes from 5°C. to -79°C. The important changes were the addition of the glycerol at 5°C. and the leaving of the spermatozoa in contact with this (now referred to as equilibration) for approximately 18 hours before deep freezing. This report encouraged workers in many countries to study these techniques, with the consequent appearance of numerous publications. Unfortunately, since comparatively few of the observations have been supported by fertility data, caution must be exercised in the interpretation of the results obtained by the post-thawing subjective motility assessments. The various techniques that have been studied are summarised in Table 10 and certain aspects will be discussed in greater detail.

B. Factors affecting the efficiency of the deep freezing process

(i) Variations in the rate of cooling. Polge and Rowson (1952) focussed attention on the possible importance of the addition of the glycerol to semen cooled to 5°C. and of leaving spermatozoa in contact with the glycerol prior to freezing (i.e. to permit equilibration), but they did not define at that time their exact rates of cooling at the different temperature stages below 0°C. However, Polge (1953) had said that this was about 1° per minute between 5° and -15°C. and 3° per minute thereafter down to -79°C. Rapid freezing by simply placing the ampoules of semen, which had been equilibrated over night at 5°C., directly into crushed dry ice was reported by Bruce (1953) to give a reasonable recovery rate; this semen was thawed out soon after deep freezing. Subsequently Bruce (1956), in a comparison of semen subjected to such rapid cooling with that deep frozen by the usual

TABLE 10

LABORATORY INVESTIGATIONS INTO DEEP FREEZING TECHNIQUES

Ref.	Modifications in Techniques Studied	Conditions giving the optimum survival rates			
		Basic Diluent	Final Glycerol Concentration	Equilibration Period	Rate of Cooling
Polge and Rowson (1952,a)	Cooling prior to glycerol addition and allowing to equilibrate.	Yolk-citrate	10%	15 to 20 hours	5°C. to -15°C. at 1° per minute then to -79°C. at 3° per minute
Blackshaw and Emmens (1953)	Inclusion of pentose sugars in diluent.	Buffered yolk-citrate	7.5%	nil	-2°C. to -79°C. in 40 minutes (in special freezing beaker)
Miller and Van Demark (1954)	Equilibration for 2,6 and 8 hours and thawing out temperature.	Yolk-citrate	7%	6 hours	5°C. to -20°C. at 1° to 4° per minute, then to -79°C. at 8° per minute.
Cragle, Myers Waugh, Hunter and Anderson (1955)	Interaction between different glycerol (2 to 11%) and sodium citrate (1.6 to 2.72%) levels and 4 to 28 hour equilibration periods.	Yolk-citrate	7.6%	14.9 hours	5° to -20°C. at 3° per minute, as fast as possible to -50°C. and then transferred to flask at -79°C.

Conditions giving the optimum survival rates

Ref.	Modifications in Techniques Studied	Basic Diluent	Final Glycerol Concentration	Equilibration Period	Rate of Cooling
Hafs and Elliott (1955)	Addition of 1% monosaccharides to non-glycerol, to glycerol, or to both fractions of diluent: Inclusion of 25% yolk in both fractions of diluent.	Yolk-citrate	7%	18 hours	5° to -12°C. at 0.8° per minute thereafter as rapidly as possible to -79°C.
Saroff and Mixner (1955)	Use of 18.4 to 27.6% yolk, 6 to 10% glycerol and 2 to 18 hour equilibration studied. Yolk contained in both parts of diluent.	Yolk-citrate	7%	18 hours	5° to -15°C. at 1° per minute thence to -79°C. at 3° or 4° per minute.

Conditions giving the optimum survival rates

Ref.	Modifications in Techniques Studied	Basic Diluent	Final Glycerol Concentration	Equilibration Period	Rate of Cooling
Graham, Erickson and Bayley, (1957)	Equilibration periods of 4, 8 and 12 hours and different cooling rates.	Yolk-citrate	7%	12 hours	5° to -15°C. at 3° per minute then to -79°C. at 5° per minute.
		Homo-genised milk	10%	12 hours	5° to 30°C. at 2° per minute and then to -79°C. at 5° per minute.

Ref.	Modifications in Techniques Studied	Basic Diluent	Conditions giving the optimum survival rates: Final Glycerol Concentration	Equilibration Period	Rate of cooling
O'Dell and Almquist (1957)	Level of and method of adding glycerol. Addition of 1.25% fructose to milk diluents, with varying equilibration periods	Yolk-citrate	7 and 10%	18 hours	5°C. to -10°C. at 0.8° per minute; to -15°C. at 1° per minute;
		Skim-milk	10 and 13%	30 minutes to 4 hours	to -20°C. at 1.5° per minute to -35°C. at 2° per minute and then to -79°C. at 4° to 5° per minute.
		Homo-genised milk	10%		

Ref.	Modifications in Techniques Studied	Basic Diluent	Conditions giving the optimum survival rates: Final Glycerol Concentration	Equilibration Period	Rate of Cooling
Jones, Perkins and Seath (1956)	Optimum glycerol levels and rate of freezing for different diluents	Yolk-citrate	7%	18 hour	5°C. to -34.4°C. at 2.2° to 3.3° per minute, then to -79°C. at 2.2° to 3.3° per minute
		Milk - chemically treated	7%	18 hour	
		Milk - heat treated	7%	18 hour	
O'Dell and Hurst (1956)	Comparison of 0.5 and 18 hour equilibration periods.	Yolk-citrate, skim-milk	8%	30 minutes but with some bulls 18 hours preferable.	5°C. to -15°C. at 0.8° per minute, to -65°C. at 3.5° per minute, then very rapidly to -79°C.

alcohol bath method, obtained 112 day non-return conception rates of 56.7 per cent and 63.0 per cent respectively, with a total of 944 first inseminations from 19 bulls. Recently, Polge and Jakobsen (1959), who used seven ejaculates from different bulls, reported similar post thawing motility rates with fast and slow cooling methods. A loss of motility, after storage at -79°C. for up to 8 weeks, was reported in semen cooled by both methods. Although the semen temperatures dropped from 0° to -79°C. in approximately 9 minutes in the fast method and in 14 minutes in the slow method, these authors found that the rate of cooling over the danger zone, i.e. -15° to -25°C., was almost identical in both methods. Luyet and Keane (1955) demonstrated that this ability of the bull spermatozoa to withstand the rapid cooling varied in different temperature ranges. They found that spermatozoa in ampoules cooled suddenly (i.e. at 20° per minute) from 0° to -27°C. and transferred after 5 minutes direct into liquid air (-195°C.) (i.e. cooled at 15° per second) showed a recovery rate of 75 per cent on thawing, whereas, if in the first stage cooling was done to only -20°C. and the semen then put direct into the liquid air, no spermatozoa survived. This suggested that in the second experiment the cooling had been interrupted at a critical temperature stage and that the spermatozoa were very sensitive to rapid cooling above -27°C. Furthermore, these results did indicate that a slowing down of the cooling process in the critical range rendered the spermatozoa relatively immune to rapid cooling below

this temperature. However, these findings were based on motility studies and they have not been confirmed with fertility trials. The need for accuracy in the measurement of temperature changes in the freezing process has not been adequately stressed. Petersen and Nordlund (1958) claimed that a thermo couple inside the ampoule gave more accurate records than either a mercury or alcohol thermometer in the freezing bath. These authors showed that alteration in the size of the ampoule could also change the freezing rate.

(ii) Presence of glycerol. Lovelock and Polge (1954), in reporting an investigation into the protective action of glycerol, pointed out that in cooling to -79°C. the spermatozoa were subjected to two hazards, one being the risk of temperature shock below -12°C. as occurs in cooling semen above zero temperature and the other being the effect of the physical changes in the medium around the spermatozoa. The latter effect was considered by these authors to be due to the exposure of the spermatozoa to excessive concentrations of electrolytes when the water in this medium changed to ice. They demonstrated that the addition of glycerol prevented this electrolyte concentration from rising to the harmful level. The period over which the spermatozoa are exposed to the unfavourable environment can be reduced by rapid cooling but then there is an increased risk of damage due to thermal shock. The glycerol, therefore, appears to act by permitting sufficiently slow cooling over the critical temperature, thereby reducing the risk of temperature shock.

The precise effect of equilibration has not been fully explained. Although it is reasonable to assume that there is a permeation of the glycerol into the cell, this has not been proved. Lovelock and Polge (1954), because they found that the spermatozoa were motile in a 15-20 per cent glycerol solution, concluded that there was some penetration of glycerol into the cells, since they considered it most unlikely that the spermatozoa would retain their motility in what would otherwise be a very hypertonic medium. Reverse movement of spermatozoa is often seen immediately after the initial dilution with glycerol and, since this disappears on storage, it could indicate that an osmotic equilibrium is established, possibly by permeation of the glycerol into the cell. The egg yolk or other constituents of the diluent may also have a protecting action against the glycerol.

(iii) Variations in the equilibration period. In studies based on the survival of the spermatozoa in semen that had been left in contact with the glycerol for only 30 minutes before being cooled below zero, Lovelock and Polge (1954) reported that the danger range in freezing semen was -15° to -20°C. However, when this was repeated using semen in contact with the glycerol for 18 hours (i.e. equilibrated) before deep freezing, Polge (1957) was unable to demonstrate a marked destruction of spermatozoa between -15° and -25°C. unless the samples were held for 30 minutes in this temperature range. The results reported by Rowson(1956), who used similar techniques, indicated that -20° to -30°C. was still

a critical temperature range for spermatozoal damage to occur and that it was important to cool over this range as quickly as possible. These findings, whilst only based on viability assessment, did indicate the possibility of an interaction between equilibration time and rate of cooling, and emphasised the need for attention to these factors. Many other workers have reported conflicting results on the effect of the length of the equilibration period on spermatozoal survival rates after deep freezing. Unfortunately, only a few controlled fertility studies have been carried out and it is difficult to assess the optimum period for this.

As a modification of the original Cambridge technique, Rowson (1953) advised that the cooling from 5° to -10°C. should take 30 minutes and the subsequent cooling to -79°C., 20 minutes. Presumably this advice was based on motility studies. Blackshaw and Emmens (1953) claimed equilibration was unnecessary when a slow rate of cooling was used provided 1.25 per cent arabinose was included in the diluent, but a subsequent fertility trial by Emmens and Martin (1957) showed that semen so treated had a lower fertility than the equilibrated semen. However, these authors concluded that the omission of the equilibration period did not significantly impair fertility, which was more affected by between bull differences, and that, provided a minimum of 30 million spermatozoa was used for insemination, the deep freezing of unequilibrated semen was justified. Subsequent work (Martin and Emmens, 1958) would appear to contradict this view since, even in the presence of fructose or

arabinose, semen, deep frozen after 1 hour of equilibration, had a significantly lower conception rate than that frozen after an 18 hour equilibration period; also in this study with the three dilution rates (30,20 and 13.3 million total spermatozoa per insemination) used the conception rates were similar. Since there would appear to be considerable advantage in being able to collect, dilute and deep-freeze the semen in one day, several other workers investigated the possibility of reducing the equilibration period. Although O'Dell and Almquist (1957), who worked with milk-diluents, reported that the inclusion of 1.25 per cent arabinose, glucose or fructose in the diluent improved the survival rates, even in the absence of the sugars equilibration periods of 30 minutes or 4 hours were preferable to one of 18 hours. O'Dell and Hurst (1956) also favoured a shortened equilibration period, especially when using a fresh skim milk diluent, but they found that several bulls required an 18 hour equilibration period for maximum spermatozoal recovery rates. Dunn, Larson and Willett (1953,c), with a limited fertility trial, and Schindler (1954,b), using viability tests, also favoured an equilibration time of 5 hours, whereas ~~both~~ Cragle, Myers, Waugh, Hunter and Anderson (1955) and Saroff and Mixner (1955) claimed that the long period of contact with glycerol (15 to 18 hours) gave the best spermatozoal survival rates. Although in the last two reports interactions between glycerol and egg yolk concentrations and between glycerol and sodium citrate concentrations were demonstrated, there was no evidence of interaction between the

equilibration time and these other variables. Graham, Erickson and Bayley (1957), using semen diluted in homogenised whole milk for approximately 2,000 first inseminations, demonstrated an overall conception rate difference of 4.4 per cent (significant at the 5 per cent level) in a comparison of semen deep frozen after 4 and 12 hour equilibration periods. In general, in this trial each bull gave the highest conception rate with the semen deep frozen after the 12 hour equilibration period, but these conception rate differences were only significant with one bull.

In the summary of certain of these laboratory studies (see Table 10) it should be noted that when maximum viability was obtained with a long equilibration period (i.e. 12 to 18 hours) the cooling rate tended to be faster than in these cases in which a short equilibration period was favoured. Also with the former the diluent was generally yolk-citrate whereas with the latter it was usually milk. It must also be pointed out that a lowered fertility is known to result when semen is stored at 5°C. for 24 hours. The conception rate which could be expected to be improved with the longer equilibration time is, therefore, likely to be also somewhat adversely affected by the storage at 5°C. prior to deep freezing.

(iv) Temperature of glycerol addition. Although in most studies the glycerol addition was made as recommended by Polge and Rowson (1952,a) at 5°C., studies by Dunn and Hafs (1953) revealed no adverse effect on motility when this was carried out at room temper-

atures. These findings were also in agreement with those of Graham, Vogt and Fisher (1958), in whose fertility trial, involving 3,005 first inseminations, 75 day non-return conception rates of 65.1 65.4 and 62.7 per cent were obtained with the addition of the glycerol-citrate at 5°,10° and 20°C. respectively, and 64.0 per cent with raw semen diluted initially in glycerol yolk-citrate at 35°C. In these investigations the semen after dilution was cooled slowly in the usual manner to 5°C. and thereafter left for an equilibration period varying from 7 to 14 hours.

The dilution of semen initially in a glycerol yolk-citrate diluent at 20°C. was reported from Reading Cattle Breeding Centre (Report, 1960,b) to give a 112 day non-return conception of 61.1 per cent with 1,176 first inseminations, compared with 58.9 per cent from 1,098 first inseminations with semen diluted initially in yolk-citrate and subsequently, after cooling, again in glycerol citrate at 5°C. In both groups the diluted semen was allowed to equilibrate for around 20 hours at 5°C. prior to ampouling and deep freezing. In contrast to the foregoing studies, Blackshaw (1955) claimed that the addition of the glycerol at temperatures above 15°C. lowered the survival rate, but it should be noted that in his work the semen had been cooled to 5°C. immediately after the initial dilution and the different portions of it warmed to the temperatures at which the glycerol additions were studied (i.e. 10°, 15° or 30°C.). This author also indicated that previous workers could possibly have attributed improved survival rates to the effect of equilibration,

whereas the effect of temperature of mixing might have been overlooked. Miller and Van Demark (1954) also indicated that it was preferable to add the glycerol at a low temperature (i.e. 4° to 5°C.). These studies were all carried out with yolk-citrate diluents. However, when using milk diluents for storage of semen at 5°C., Almquist (1959) obtained the best survival rate when the glycerol was added after cooling the diluted semen to 5°C. In the recent report by Polge and Jakobsen (1959), the best survival rates were obtained with semen frozen at 7 hours after dilution when the glycerol was added either at the initial dilution, (i.e. at 25°C.) or after cooling to 2°C. (i.e. 2 hours after the initial dilution). In this study the survival rates were similar irrespective of whether the semen was left in contact the glycerol for 30 minutes or for 3 hours. It was suggested that the protective effect depended on the length of time that the semen had been cooled and diluted before being frozen, and not on the actual length of time that the spermatozoa had been in contact with the glycerol. However, no fertility studies were reported in this paper and, since these results, from only seven ejaculates, are in contradiction to those in previous reports (Polge and Rowson, 1952,a; Polge, 1953), their significance cannot be properly assessed until they have been confirmed in fertility trials involving several bulls. The necessity for having the two diluent fractions at the same temperature has, however, been accepted by all workers.

(v) Method of glycerol addition. The addition of the glycerol-

containing portion of the diluent in three parts at 10 minute intervals was reported by Miller and Van Demark (1954) to give better recovery rates than adding the glycerol fraction at one step. This has also been recommended by Almquist (1959) when using milk-glycerol diluent for storage of semen at 5°C. De Groot (1955) and Macpherson (1958) described an apparatus that automatically added the glycerol to the diluted semen. Rowson (1956) claimed that, although in most cases the rapid addition of glycerol does little harm, in others its adverse effect is made quite noticeable by the numbers of backward-moving spermatozoa appearing after its addition. He advocated the introduction of glycerol by dialysis through a cellophane membrane but there are no reports of this being used in practice.

(vi) Diluent composition.

(a) Glycerol

(1) Glycerol concentration. Most studies on this, the most important constituent of the freezing diluent, have been concerned with determining its optimum concentration. Polge and Rowson (1952,b), in a small scale experiment, demonstrated that 15 per cent glycerol in the standard egg yolk-citrate diluent did not impair the fertilising capacity of bulls semen stored at 5°C. Holt (1953,b), in a split sample field trial involving 6,000 first inseminations, showed that the inclusion of 10 per cent glycerol in semen diluted in the egg yolk-citrate diluent, and stored at 5°C., resulted in a 4.3 per cent increase in the 3 month non-return conception rate compared with that of semen in yolk-citrate alone. The increased conception rate occured with 4 of the 9 bulls used in the trial.

Evidence that this effect of the glycerol in storage at 5°C. may depend on the concentration of the egg yolk in the diluent was reported from the Reading Cattle Breeding Centre (Report, 1954,b) where, with 10 per cent glycerol and 50 per cent yolk in the diluent no effect on conception rate was noticed; whereas, in a subsequent experiment (Report 1956,b), in which 25 per cent egg yolk was used, there was an increased conception rate in the presence of the 10 per cent glycerol. These fertility trials were therefore of fundamental importance since they demonstrated that glycerol at a concentration of 10 to 15 per cent did not adversely affect the fertility of semen stored at 5°C. For use in deep frozen semen, Polge (1953) reported that a concentration of 10 per cent glycerol in the diluent was the highest level that could be used without damaging the spermatozoa, as judged by motility observations, and De Groot (1952) reported that 7.5 per cent glycerol gave best results in motility studies. Fertility data presented at the Society for the Study of Animal Breeding Symposium (Report, 1953,e) indicated that most British workers were using a final concentration of 10 per cent glycerol in the diluent, but subsequent reports have tended to favour the lower concentration of glycerol.

(2) Interaction between glycerol and egg yolk levels.

Saroff and Mixner (1955), using post-thawing motility ratings, demonstrated a marked interaction between the levels of egg yolk and glycerol in the deep freezing diluent. They recommended the inclusion of egg yolk in the glycerol-citrate fraction of the diluent; hitherto workers had added glycerol-citrate alone, all the egg yolk being included in

the initial egg yolk-citrate diluent. The optimum spermatozoal survival rates were obtained with final concentrations of 20 per cent egg yolk and 7 per cent glycerol; the glycerol containing fraction of the diluent was added in three parts. These workers suggested that the egg yolk reduced the effectiveness of the glycerol and, therefore, with a high egg yolk percentage a high glycerol percentage was required. This observation is in accord with the fertility results reported above when semen diluted in a glycerol yolk-citrate diluent was stored at 5°C.

In a fertility trial Hafs and Elliott (1955) compared the effect of the inclusion of 50 per cent yolk in the non-glycerol fraction with the use of 25 per cent yolk in both the glycerol and non-glycerol fractions as above; with 1,515 first inseminations the 60-90 day the non-return conception rates were 59.7 per cent and 68.0 per cent respectively, these being significantly different ($p<0.01$).

(3) Interaction between glycerol and citrate levels.

Cragle et al. (1955) using a final egg yolk concentration of 24 per cent noted an interaction between glycerol and citrate levels; on the basis of motility studies they reported that the optimum final levels of sodium citrate and glycerol were 2.9 per cent and 7.6 per cent respectively, and that, although there were variations in the motility ratings with semen from different bulls deep frozen in the same diluent, the variation between the bulls was of the same order in the different diluent variations studied. Final concentrations of sodium citrate, between 2.4 and 3.3 per cent, and of glycerol between 4.5 and 8 per cent, were found to give the best motility ratings on thawing out after deep

freezing. However, Kinney and Van Demark (1954), who also used motility studies to observe the effect of varying both the egg yolk and citrate concentration in the presence of 7 per cent glycerol (final concentration), found that, with an initial fixed citrate concentration, the optimum final range of egg yolk was 16-24 per cent and the optimum final concentration of citrate was between 1.55 and 1.95 per cent (using sodium citrate dihydrate). These concentrations were obtained by diluting the semen inttially in a diluent containing equal volumes of egg yolk and 2.9 per cent sodium citrate solution and subsequently in an equal volume of a 2.9 per cent sodium citrate solution containing 14 per cent glycerol. Polge (1957) also recommended the use of a diluent containing final concentrations of 2 per cent citrate, 25 per cent yolk and 7.5 per cent glycerol, but no fertility data were given to support this. Most workers in the U.S.A. appear to be using a diluent of such a composition. (Erickson, Graham and Frederick, 1954; Kinney and Van Demark, 1954; Saroff and Mixner, 1955).

(4) Glycerol level in milk: O'Dell and Almquist, (1957) and Erickson et al. (1954) reported that with milk diluents 10-13 per cent glycerol is required; Macpherson (1955) used a 10 per cent glycerol concentration in homogenised milk, but Jones, Perkins and Seath (1956) reported that 7 per cent glycerol gave the best survival rate using an 18 hour equilibration period. Amann and Almquist (1957), with motility studies, found that, with skim milk, the glycerol level required to be adjusted according to the total solids content of the skim milk, for example, with fresh skim milk containing

9 per cent solids 11 per cent glycerol gave optimum recovery rates, whereas, with 11 per cent solids 13-15 per cent glycerol gave better results.

(5) Variation in glycerol composition and impurities. Little attention has been paid to possible variations in the glycerol but Myrendahl (1954), in an investigation into a fall in conception rate with deep frozen semen, demonstrated an adverse effect on spermatozoal survival and on subsequent fertility with certain brands of glycerol. Although the fact that the survival rates were assessed by live/dead staining lessened the importance of these findings (see page 210), this observation on the possible effect of impurities in the glycerol could be of some importance, but it has not been reported by other workers.

(b) Egg yolk content. Apart from the actual concentration of egg yolk and its possible interaction with the glycerol diluent, as discussed above, it has been generally assumed that the yolk acts as in the fresh semen diluent. However, Bialy, Ludwick, Hess and Ely, (1957) reported in detail on a method of extracting the lipo-protein from egg yolk. The addition of 5 per cent of this to the freezing diluent gave a highly significant increase in spermatozoal survival, but 2.5 per cent had no effect and 10 per cent did not give a higher recovery rate than the 5 per cent addition. Blackshaw (1955) showed that egg yolk plus lecithin gave best spermatozoal revival rates when compared with egg yolk alone and milk plus lipo-protein.

(c) Citrate content. The optimum concentrations of this have been referred to above, but it should be noted that Australian

workers (Emmens and Martin, 1957) made particular reference to the use of a buffered citrate solution. (80 parts of a 3 per cent sodium citrate dihydrate solution plus 20 parts of an 0.1 M sodium phosphate buffer to ensure a pH value of 7.0)

(d) Milk. Homogenised whole milk is apparently being used successfully in Canada (Macpherson, 1954), and fertility data with this have also been reported from the U.S.A.(Williams and Green, 1956; Graham et al. 1957), O'Dell and Almquist (1957) carried out motility studies with chemically-treated (i.e. with cysteine) and heat-treated homogenised whole milk and skim milk diluents; they reported on the optimum glycerol concentrations and equilibration times required with these diluents. They claimed that the addition of 1.25 per cent fructose improved the recovery rates but, unfortunately, no fertility trials were carried out to test the practical value of this addition.

Bruce (1956), in a pilot field trial, found that a diluent containing a final concentration of 10 per cent skim milk powder and 10 per cent glycerol gave a conception rate of 58.7 per cent compared with 56.7 per cent with the usual yolk-citrate diluent over 1,066 first inseminations. However, in a more extensive trial of these diluents (Reading Cattle Breeding Centre, Report, 1958) a marked reduction in fertility occurred with a similar skim milk powder diluent. Recently Erickson and Graham (1959) inseminated approximately 900 cows with semen stored in each of the following diluents, yolk-citrate, homogenised milk, skim milk, yolk-glucose-glycine, and the 75 day non-return conception rates were 64.7, 66.2, 65.3, and 41.3 per cent,

respectively. In this trial, therefore, the milk and yolk diluents were equally satisfactory but the yolk-glucose- glycine had adversely affected the conception rates.

(e) Use of anti-bacterial substances. The use of 0.3 per cent sulphanilamide by Dunn, Larson and Willett (1953,d) in the diluent resulted in a marked reduction in spermatozoal viability. These authors recommended that whilst sulphanilamide should not be included in the freezing diluent, the use of antibiotics such as streptomycin and penicillin should be investigated in fertility trials. Erickson et al. (1954) found that in both milk and yolk diluents streptomycin and penicillin had no adverse effect on motility. It is now the usual practice to include these antibiotics at concentrations of 500 or 1,000 µg/ml. in the freezing diluents (see page 216).

(f) Inclusion of sugars. Emmens and Blackshaw (1950), working primarily with ram semen, reported that the inclusion of 1.25 per cent arabinose, rhamnose or xylose in the yolk-citrate diluent plus phosphate improved the recovery rates; Blackshaw and Emmens (1953), Emmens and Martin (1957) claimed that in the presence of these sugars the equilibration period could be shortened or dispensed with. Hafs and Elliott (1955) studied the addition of 1 per cent fructose, glucose or xylose and found that the fructose addition gave the best recovery rates but not markedly so. Although it was claimed that the inclusion of the sugars was of practical importance, since it permitted a reduction of the equilibration period, in the motility study reported above, (O'Dell and Almquist, 1957) any beneficial effect of including sugar in milk diluents was not necess-

arily associated with a shortening of the equilibration period. This was confirmed also in fertility studies by Martin and Emmens (1958), using yolk-citrate diluents with arabinose or fructose.

(g) Surface tension reducing agents. The inclusion of these in the glycerol fraction of the diluent was studied in vitro. Of the 5 agents tested "Tween" 80, at 1 in 40,000 (v/v. final dilution), gave best results as judged by the motility on thawing after storage at -79°C. for 7 days. Even in this the reduction of the percentage of motile spermatozoa was greater than in the controls but no fertility trials were reported (Fosgate, Aschbaker, Smith and Tyler, 1957).

(vii) Dilution rates. Within certain limits, the number of motile spermatozoa and, therefore, also the dilution of the semen, could be expected to have an important bearing on fertility after deep freezing. Polge and Rowson (1952, a) used very low dilution rates but, subsequently, Polge and Rowson (1952,b), using a small number of cows (11 per group), obtained a satisfactory conception rate with dilution rates up to 1:90. Bruce (1956), using a yolk-citrate-glycerol diluent over 2,462 first inseminations to compare 1:20 and 1:50 dilution rates, obtained 112 day non-return conception rates of 55.2 per cent and 52.9 per cent, respectively.

Bratton, Foote and Cruthers (1955) found no marked difference between the fertility of unfrozen semen, containing 10 million motile spermatozoa per 1 ml., and that of semen frozen in single ampoules containing 8 to 14 million motile spermatozoa per 1 ml. ampoule. In a more extensive split sample trial, involving 3,500 first inseminations from 8 bulls (Bratton, Flood, Foote, Wearden and Dunn 1957), the respect-

ive 60 to 90 day conception rates were 71.0, 73.2 and 69.8 per cent for semen stored at 5°C.for one day, at -79°C. for 1 week and at -79°C. for 17 weeks. These conceptions rates differences were not significant and within treatments,although the number of observations were limited, there were no significant correlations between numbers of motile spermatozoa and fertility; these averaged in the unfrozen semen 8.6 million, in the frozen semen stored for 1 week 11.6 million and in the frozen stored for 17 weeks 9.3 million motile spermatozoa per ml of diluted semen. There was, however, a falling off in the post-thawing motility rating after storage at -79°C. for 17 weeks. In contrast to this Dunn, et al. (1953,c), using frozen semen containing 20 million spermatozoa per ml., obtained a 60 to 90 day non-return conception rate of 56 per cent with 287 first inseminations and a 70 per cent rate with unfrozen semen from the same collections. Recently, Erickson and Graham (1959) found that diluted samples containing initially 30 million living spermatozoa per ml. gave a significantly higher conception rate than those containing 10 million living spermatozoa per ml. (72.7 per cent compared with 63.1 per cent). These workers made no reference to the density of the undiluted samples or to the dilution rate of the semen, but they suggested that at least 20 million live spermatozoa per insemination were required for satisfactory fertility. Although this increased fertility was claimed to be due to the higher number of living spermatozoa inseminated (21 million compared with 7 million), since Bratton et al. (1957) obtained over large numbers of inseminations comparable deep frozen and

fresh semen conception rates with relatively low counts of motile spermatozoa, it would appear that the quality, as well as the actual numbers, of spermatozoa is of importance. Martin and Emmens (1958) obtained similar conception rates when using 30, 20 or 13.3 million total spermatozoa per insemination.

(viii) Method of ampouling. Most centres have adopted the use of single dose sealed glass ampoules for semen storage. Although the usual practice is to ampoule after the equilibration period and just prior to deep freezing, this may not be necessary. With semen ampouled at the beginning of the equilibration period at the Reading Cattle Breeding Centre (Report, 1959,b) a 112 day non-return conception rate of 64 per cent was obtained with 1,216 first inseminations, with control semen ampouled after equilibration a 61.3 per cent conception rate was obtained with 1,237 first inseminations. The Milk Marketing Board (Report 1954,a) reported on the use of a cork covered with a synthetic sealing compound, but with these there is a risk of alcohol leakage into the ampoule as happened with the rubber stoppered vial (Macpherson, 1954). A similar difficulty was experienced by Van Demark and Kinney (1954,b) who, when using plastic ampoules, found great difficulty in sealing to prevent alcohol leakage. However, Dunn, Hafs, Buckner, Young, Conrad, Willett and Larson (1954), using alcohol/dry ice storage, obtained a 60 to 90 day non-return conception rate of 62.3 per cent with 313 first inseminations with semen stored in polythene bulbs, compared with 60.7 per cent with 298 first inseminations with semen contained in the usual glass ampoules. Musgrave and Heath (1957) found that the post-thawing motility of

semen stored at -79°C. for 40 to 90 days in glass containers was superior to that stored in plastic containers. No reason was put forward for this difference. Murdisk (1958) reported on the detection of isopropyl alcohol in frozen semen stored in polythylene ampoules. However, in a fertility study involving 727 first inseminations Brugman and Schmidt (1958) obtained 60 to 90 day non-return conception rates of 59.9 per cent and 56.0 per cent with semen stored in plastic and glass ampoules respectively, these differences were not significant. In a recent report (Graham and Erickson, 1959), the overall 75 day non-return conception rates were 65.4 per cent with 1,607 first inseminations with semen stored in glass ampoules, and 58.9 per cent with 1,711 first inseminations with semen held in plastic ampoules. In each of the four parts of this investigation the glass appeared to be superior to the plastic ampoules although the fertility differences were not always significant. Also storage in either dry ice alone or in dry ice/alcohol mixture did not effect fertility with either the glass or, what is most important, with the plastic ampoules. The satisfactory storage of semen deep frozen in straw like containers inside plastic bags was reported by Jakobsen (1956). In a study of the method of filling and sealing the glass ampoules Erickson and Graham (1959) demonstrated no fertility differences with semen ampoules filled by either the gravity or force-feed methods; however, the semen from pull-sealed ampoules had a significantly higher conception rate than that from the tip sealed ampoules, possibly this could have been due to the greater amount of heat required in the latter method, adversely affecting

the spermatozoa.

In order to reduce the time consuming, and therefore expensive, process involved in ampouling each single dose, Bratton et al. (1955) tried deep freezing semen at a low dilution rate (200 x 10^6 motile spermatozoa per ml. diluent) using 1.1 ml. in each ampoule; this was thawed out in the laboratory immediately after freezing, diluted further in diluent at 5°C. to contain 10 x 10^6 motile spermatozoa per ml. and dispatched for use in the field 24 to 60 hours later. It gave a 60 to 90 day non-return conception rate of 51.7 per cent with 449 first services, compared with conception rates of 74.5 per cent with semen stored unfrozen at 5°C. for 1 to 3 days, and 75.4 per cent with 147 first inseminations with semen frozen in single ampoule doses, stored at -79°C. for 1 to 103 days and thawed just prior to insemination. Although this was not an extensive investigation it did indicate that deep frozen semen once thawed out, even although it was stored at 5°C., could only retain its fertilising capacity for a very short time.

(ix) Method of freezing. For small quantities of semen this can be readily carried out by cooling the alcohol in a thermos flask by the addition of small particles of solid CO_2., but when large numbers of ampoules are to be frozen in one batch, a mechanical freezing apparatus is a necessity. Equipment of this type was described by Ehlers and Rice (1957); cooling is achieved by the use of cold flowing alcohol, the temperature of which can be quickly reduced. The proper and rapid circulation of the alcohol within these freezing tanks would

appear to be just as important as, for example, the circulation in a water bath for use at high temperatures. Henderson, Macpherson and Snyder (1956) described an electrically powered freezing and storage apparatus in which the cooling was achieved by the circulation of cooled methylene chloride. Automatic cooling methods were also reported by Polge and Lovelock (1952) who immersed a special polythene beaker, containing the ampoules, in an alcohol dry ice mixture, and by Graham and Marion (1953) who placed an insulated metal tank containing the ampoules into an acetone bath at -79°C. These methods have not been extensively used but a modification of the polythene beaker apparatus was reported from Australia (Blackshaw, Emmens, Martin and Heyting, 1957,a)

C. Storage methods

Whilst a temperature of below -70°C. can be readily maintained by a dry ice alcohol mixture in a thermos flask, this method of storage, although very efficient, is only suitable for a limited number of ampoules as it requires daily checking, and the ampoules from different bulls cannot be readily identified in this. Many types of insulated storage boxes have been developed. The original type, described by Rowson and Polge (1953), had a front opening cabinet, but this was of faulty design since opening it allowed the cold air to escape. Even the top-opening cold air type of store, which merely contains solid CO_2, is also unsatisfactory for storage at -70°C. in view of the risk of a rise in temperature. Unless mechanical refrigeration is used, the most suitable method for storage at -70 to -79°C. is the large alcohol-filled insulated tank in which the semen

is stored in trays arranged in racks; the dry ice, which is also immersed in alcohol, is stored in perforated compartments on two sides of the semen storage compartment; such a cabinet was described by Swanney (1953). By this means it is possible to handle the semen ampoules, while they are immersed in a tray of alcohol, at the same temperature as the rest of the bank. Rowson (1956) pointed out that if such a cabinet is left open the temperature is not so likely to rise, provided there is ample solid carbon dioxide present.

Mechanical refrigeration was claimed by Etgen, Ludwick, Richard, Hess and Ely (1957) to be more effective than the dry ice storage cabinets, as judged by percentage of live spermatozoa on thawed out samples after storage for 24 weeks. The use of mechanical refrigeration has been adopted by several organisations in the U.S.A. and Canada (Henderson et al. 1956), but so far, largely on the grounds of costings, this has not been used in Great Britain. Rowson (1956) claimed that a temperature lower than -79°C. might be necessary where storage was required for longer than 6 to 7 years, and advocated the use of liquid nitrogen or liquid oxygen. The latter is dangerous to handle, and, therefore, its use has not been developed, but large insemination organisations in the U.S.A. now use liquid nitrogen containers for routine storage. In limited trials, with semen stored for relatively short periods at -196°C. in liquid nitrogen, Larson and Graham (1958), Pickett, Jones, Heller, Cowan and Gosslee (1959) obtained conception rates similar to those got with semen stored at -79°C.

D. Thawing out of semen and general handling in the field

Although Polge and Rowson (1952,a) did this in a water bath at 40°C., Rowson (1953) was unable to demonstrate any significant difference in live-dead spermatozoa counts of stained smears prepared from semen samples thawed out in boiling water, water at +90°C. and water at 5°C., but the nigrosin eosin staining technique, used for this assessment, has been found to give varying results. (see page 210) Hafs and Elliott (1954) obtained higher motility ratings with semen thawed in a water bath at 40°C. than with that thawed at 1°C., but intermediate temperatures gave the lowest recovery rates. However, in fertility studies involving 661 first inseminations, no significant differences were obtained with semen thawed at 5°C., 20°C. or 40°C. O'Dell and Almquist (1954) and more recently, Brugman, and Schmidt (1958) found no significant difference in motility after thawing at 5° or 38° to 40°C., but many other workers had previously expressed a preference for thawing at 5°C. (Snyder, Rutz and Marian, 1955; Van Demark and Kinney, 1954,b; Bratton et al. 1955). It does appear that this is one important technical point which requires to be critically studied in fertility trials. Bruce (1953) logically claimed that by thawing with tap water on a farm the temperature of the thawed-out semen, especially in winter, would then approximate fairly closely the atmospheric temperature. The thawing out of the semen some time prior to insemination has already been referred to as giving a lowered conception rate (Bratton, et al. 1955).

E. Assessment of semen quality after thawing

As with semen stored at 5°C. the prediction of the potential

fertility of a given sample of semen is not readily possible. Mixner and Saroff (1954) demonstrated an interference of glycerol with the usual differential staining technique for the assessment of the live/dead spermatozoa rates. With glycerol levels above 4 per cent, the proportion of dead (i.e. stained) spermatozoa appeared to increase disproportionately to the estimated number of motile spermatozoa. It was suggested that the higher glycerol levels increased the permeability of the spermatozoa and it was concluded that the live dead staining could not be reliably used in frozen semen assessment. In contrast to this, Blackshaw (1958) indicated that glycerol (up to a concentration of 15 per cent) did not appear to have any marked effect on live/dead estimates with eosin or congo red stains. Only the assessment of post-thawing motility has been generally used. Although this is a subjective assessment, a reasonable degree of accuracy can be got by microscopic examination of a thin smear under the high power (1/6") objective under standard conditions.

In a study of rapid freezing rates O'Dell, Almquist and Marsh (1958) found a need for re-examining the samples after 10 days' storage at -79°C. The greatest decline in motility in storage was reported to occur in the first 4 weeks of storage regardless of diluent or freezing rates. These findings emphasised the need for caution in measuring the effect of changes in technique by a single post-thawing motility assessment.

E. Metabolism of deep frozen semen

A series of metabolic studies on semen, which had been deep

frozen and stored at -79°C. were reported by White, Blackshaw and Emmens (1954). Semen diluted in a phosphate-fructose diluent with and without the addition of glycerol and arabinose showed little metabolic activity when thawed out and incubated at 37°C. Equilibration in the glycerol-arabinose diluent gave similar results, but after the addition of egg yolk to the diluent there was an increase in the oxygen uptake of the thawed out semen. Although these workers demonstrated that glycerol and arabinose could be oxidised by bull spermatozoa, and that lactic acid was produced by the spermatozoa from glycerol but not from arabinose, they considered it unlikely that glycerol could play an important part in the metabolism of the spermatozoa at ultra-low temperatures, even if such was taking place. O'Dell and Almquist (1958) confirmed that deep freezing markedly reduced the metabolic activity of the thawed out spermatozoa, but the depression of lactic acid production, due to freezing, was lower in semen stored in a milk/glycerol than in a yolk/glycerol diluent. They found no indication of any respiratory changes over 6 months storage at -79°C. Evidence was presented that the reduction in metabolism was relatively greater than the loss of motility as a result of deep freezing. The higher rate of metabolism after thawing with the milk diluent may be a reflection of the level of available utilisable substrate and not an indication of a greater protective action of the milk diluent. Also this cannot be taken to indicate that a higher conception rate can be expected with milk than with yolk-citrate diluents.

G. Long term storage

Certain motility studies have indicated a dropping off in spermatozoal survival after storage. (Dunn and Hafs, 1956; Buch, Smith and Tyler, 1956); Rowson (1956) estimated that there was a 15 per cent spermatozoal mortality per year of storage, but no data were given on how this estimate was made and no corresponding effect on fertility was reported. Long term storage fertility studies have been carried out at the Reading Cattle Breeding Centre, (Report, 1960,b) where no effect on conception rate has been demonstrated with semen diluted 1:20 in yolk-citrate-glycerol and stored in a dry ice alcohol mixture for 4 years. (see Table 11)

TABLE 11

EFFECT OF LONG TERM STORAGE OF SEMEN ON CONCEPTION RATES

	Length of storage period prior to use for insemination							
	1 - 4 weeks		1 year		2 years		4 years	
BULL	First Insemin-ations	Per cent conce-ption	First Insemin-ations	Per cent conce-ption	First Insemin-ations	Per cent conce-ption	First Insemin-ations	Per cent conce-ption
S.3	105	67.6	129	64.8	142	66.1	133	63.2
S.14	104	65.4	143	59.5	156	62.8	143	60.8
TOTAL	209	66.1	272	61.8	298	64.4	276	62.0

Note. Conception rates were assessed on a 112 day non-return basis.

Cambridge workers, with semen at a low dilution (1:4) and with smaller numbers of inseminations, found no effect on fertility after storage for over 4 years (Rowson, 1956). Similarly Macpherson (1956),

also with a limited number of inseminations, found that semen from 5 bulls diluted 1:40 in a milk-glycerol diluent was still fertile after 3 years storage. In both these studies it was claimed that fluctuations of storage temperatures between -79° and -68°C. were reflected in the quality of the semen when thawed out.

Mixner and Wiggin (1957), reporting similar fertility studies with 102 first inseminations, found no drop in fertility after 2 years' storage at -79°C. It is noteworthy that in this study, in which 5 bulls were used, the 60 to 90 day non-return conception rate was 65.7 per cent with 102 first inseminations with semen stored for 2 years whereas with semen from the same collections, used fresh two years previously, the conception rate was 68 per cent with 175 first inseminations.

H. Variation between bulls according to freezability and possible effect of spermatozoa maturity and seminal plasma

Milovanov (1934), demonstrated that the spermatozoa of different bulls show variation in their resistance to hypertonic solution, and, if the theory, put forward by Lovelock and Polge (1954), that the glycerol acts by preventing a sudden increase in the osmotic pressure of the fluid around the spermatozoa is correct, it would be logical to expect variations in the ability of the spermatozoa of different semen samples from different bulls to withstand the deep freezing process, owing to the variation in the semen itself. Polge (1953) claimed that by strict attention to techniques, this should not be a serious problem, but Rowson (1953) and Swanney (1953) also noted variations between bulls

on laboratory motility assessments after thawing out. This was also confirmed in more extensive motility studies by Dunn, Hafs and Young (1953,b), who found significant differences between bulls but not within bulls. The other factors, which would have to be considered as possibly having an influence on the above results, would be the density of the semen samples and the maturity of the spermatozoa. Rowson (1953) and Holt (1953,b) reported that with dense semen samples the survival rates after deep freezing and thawing were lower than with less dense samples. Several workers have studied the possible effects of stage of maturity and of the seminal plasma on the deep freezing process. Kinney and Van Demark (1954), in studies on the possible effect of maturity, collected 20 consecutive semen samples from two bulls over a 4 hour period; the percentage of spermatozoa surviving freezing, as assessed in post-thawing motility studies, was found to increase up to the fifth ejaculate only and thereafter there was a decrease; epididymal and washed spermatozoa were found to be the least resistant to the freezing process. Ohms and Willett (1955) found that the second ejaculate gave a significantly higher recovery rate than did the first ejaculate. Subsequently, Willett and Ohms (1958,a) centrifuged the semen samples and, after studying the effect on the motility of interchanging the seminal plasma, they concluded that the superiority of the second ejaculate was due to the spermatozoal cell itself and not to the seminal plasma. These authors noted no difference in freezability of semen collected, after slaughter, from the caput and cauda epididymis and the ampulla of the ductus

deferens. O'Dell, Almquist and Amann (1959,a), who investigated the suitability for freezing of successively collected ejaculates, found no significant differences between the first five ejaculates. In view of their findings with post-thawing motility studies they recommended that "with adequate sexual preparation, the collection of 6 or 7 consecutive ejaculates from a bull for deep freezing should be satisfactory". These workers used only samples showing an initial motility of at least 30 per cent and a minimum of 150 million motile spermatozoa; the final dilution contained 15 million motile spermatozoa per ml.

Provided that the bull is in regular use, it would appear that no difference should be found in the successive ejaculates which show an initial satisfactory motility. There is also no clear evidence that the seminal plasma has any effect on the resistance of the semen to the deep freezing process. Brugman and Schmidt (1958) reported having obtained a conception rate of 65.4 per cent with semen deep frozen from a second ejaculate and 50.2 per cent from a first ejaculate, but no details were given as to the number of first inseminations on which these results were based.

I. Freeze drying of bovine spermatozoa

Although this is now used extensively for the storage of bacteria at room temperatures, attempts to store spermatozoa under similar conditions have not been successful (Leidl, 1956; Bialy and Smith, 1957; Sherman, 1957; and Albright, Erb and Ehlers, 1958,b).

Leidl (1956) indicated that glycerol, although essential for deep freezing, could adversely affect freeze drying since it prolonged

the drying process and could also have a toxic effect on the spermatozoa. In the later work of Juscenko (1957, 1959) the spermatozoa, after undergoing an equilibration period in a glycerol diluent, were separated off by centrifuging, transferred to a freone heptane mixture, frozen to -78°C. and dried for 10 - 18 hours in vacuo; this method was said to be aimed at the prevention of crystal formation within the spermatozoa and in the surrounding fluid in order that the spermatozoa could be frozen into a solid state in which the drying could be carried out without any osmotic pressure changes occurring. By this procedure the best motility rating of the reconstituted semen was 15 per cent; no fertility studies were reported with bull spermatozoa so treated, but pregnancies were established with rabbit semen which had been similarly freeze dried. However, after freeze drying bull semen, diluted in only yolk-citrate diluent and taken up on a nylon gauze, in some experiments a 40 to 50 per cent motility rating was obtained when the semen was reconstituted within 8 hours after freeze drying (Merryman and Kafig, 1959). One pregnancy has been established from semen freeze dried in their laboratory, indicating this method of preservation could yet prove to be feasible (Merryman, 1960).

J. Effect of deep freezing on pathogenic organisms

Macpherson and Fish (1954), using suspensions of Brucella abortus, Listeria monocytogens, Cornebacterium pyogenes, and Vibrio fetus, found, by cultural methods, that these organisms were all viable after deep freezing in the usual diluents, irrespective of whether the usual anti-biotics (i.e. penicillin and streptomycin) were included or

not. Morgan, Melrose and Stewart (1959) confirmed these findings when using suspensions of V. fetus cultures alone, and also recovered this organism from each of two maiden heifers inseminated with deep frozen V. fetus-infected semen in a diluent containing penicillin and streptomycin. Certain protozoan parasites have also been shown to be resistant to the deep freezing process. Fulton and Smith (1953) reported that Entamoeba histolytica resisted - 79°C. in the presence of glycerol but with Trichomonas foetus the reports are conflicting. Joyner (1954) failed to recover living trichomonads after equilibrating in 10 per cent glycerol for 16 hours, using both yolk-citrate and milk diluents, and subsequent freezing to -79°C. Rowson (1956), on repeating these observations, was able to recover these organisms alive. Similar results were reported by Leidl and Mahrla (1954), by Mc.Wade and Williams (1954) and by Blackshaw and Beattie (1955), who also confirmed that T. foetus survived freezing at -79°C. It would appear that when freezing is carried out immediately after the glycerol addition the trichomonads will survive, but if they are exposed to glycerol for the usual long equilibration period their chances of survival are markedly reduced. In the case of V. fetus there appears to be little doubt that this organism can remain viable and infective in deep frozen semen. However, Mc. Entee, Gilman, Hughes, Wagner and Dunn (1959), following studies with V. fetus infected semen deep frozen in the presence of penicillin and streptomycin, suggested that there was little risk of spread of the infection provided the semen was held in store for at least one week after deep freezing, but the reason for this

is not clear.

K. Conception rates with deep frozen semen

Although numerous workers have based their investigations on post-thawing motility studies for assessment of results, it is surprising how relatively few controlled fertility trials have been reported, especially since such trials are the only reliable means of assessment of any changes in technique. Whilst deep frozen conception rates comparable with those obtained with fresh semen (i.e. stored at 5°C.) have been reported, many workers would agree that under routine conditions a lowered conception rate is often obtained. Some of the reported variations in fresh and frozen semen conception rates are shown in Table 12.

Although there is a risk, as pointed out earlier, that under faulty conditions of storage the fertility of deep frozen semen may decline and, therefore, account for some of the conception rate differences reported above, at present a lowering of the conception rate by between 5 per cent and 10 per cent can be expected when using deep frozen semen irrespective of its length of storage. However, for a strict comparison, the fertility should be compared with that of fresh semen used 24 hours after collection, in order to allow for loss of fertility during the equilibration period; in such a comparison Madden (1956) found no difference between conception rates with 24 hour old fresh and deep frozen semen.

L. General remarks on usefulness of deep frozen semen

The need for the extended use of proven sires is considered to be sufficiently important to warrant the use of deep frozen semen, even although it may well result in a lowered conception rate. With frozen semen

constantly available, planned matings with special merit sires and a nominated service from particular bulls can be readily arranged; also having semen in store from a valuable bull is an insurance against death or injury rendering the bull unfit for normal use in artificial insemination or natural service. Long distance shipments can now be readily accomplished by this process and under normal insemination conditions this can be used to provide a service for minority breeds with a single bull, thereby considerably reducing overhead costs. Private breeders have also extensively used the storage of semen under deep freeze to control and/or prevent spread of venereal infection within their own herds, ~~and~~ to meet requests for service by their bull in other herds without exposing their bull to the risk of infection from these outside herds and to extend progeny testing. Although the costs of storage and of processing of deep frozen semen have been reported to be relatively greater than those for fresh semen stored at 5°C. (Dunn and Kimpland, 1959), in the U.S.A. several centres have switched over entirely to the use of deep frozen semen. Semen from bulls of the temperate breeds can, by this means, be made available for insemination of cattle in tropical areas, thus obviating the expense of transport and of the risks to the health of such bulls under tropical conditions.

One major difficulty, in this connection, is that dry ice is generally not readily available in tropical areas for the maintenance of the storage bank; therefore, unless a regular supply of this

can be arranged, or a reliable electrical refrigeration unit made available, the use of deep frozen semen should not be attempted.

TABLE 12

CONCEPTION RATES WITH FRESH AND DEEP FROZEN SEMEN

Ref. (1)	Semen storage conditions	First Inseminations	Non-Return Conception Rate (2)
Dunn, Hafs, Buckner, Young, Willett, Conrad and Larson (1954)	Fresh, stored 24 hours at 5°C.	6,663	71.0 (a)
	Deep frozen and stored 14 days at -79°C.	2,163	59.0
Reading Cattle Breeding Centre (Report, 1956,b)	Fresh, used on day of collection	670	61.6
	Fresh, used 24 hours after "	626	58.8
	Total	1,296	60.2 (d)
	Deep frozen	1,620	48.4
Emmens and Martin (1957)	Fresh, at 5°C. for up to 54 hours	533	59.3 (c)
	Deep frozen at -79°C. — without equilibration	514	56.2
	Deep frozen at -79°C. — after equilibration 18 hours	513	63.7
	Total	1,027	60.0
Williams and Green (1956)	Fresh, at 5°C.	979	73.9 (a)
	Deep frozen at -79°C.	1,207	65.3
Bratton, Foote and Cruthers (1955)	Fresh, at 5°C. for 24 hours to 60 hours	459	74.5 (a)
	Deep frozen (-79°C.), stored 1 day, thawed diluted in laboratory and sent out into field	449	51.7
	Deep frozen (-79°C.), stored 1 day, thawed at farm	55	72.7

Ref. (1)	Semen storage conditions	First Inseminations	Non-Return Conception Rate (2)
cont....	Deep frozen (-79°C.), stored 103 days, thawed at farm	92	77.0
Y			
Madden (1956)	Fresh, at 5°C. for 3-10 hours	51,848	67.9
	Fresh, at 5°C. for 27-34 hours	15,643	62.2 (e)
	Deep frozen stored up to 2months at -79°C.	17,433	60.5
	Deep frozen stored over 2 months at -79°C.	13,352	58.0
Bratton, Flood, Foote, Wearden, and Dunn (1957)	Fresh, at 5°C. for 1 day	1,278	71.0 (a)
	Deep frozen stored for 1 week at -79°C.	1,151	73.2
	Deep frozen stored for 17 weeks at -79°C.	1,094	69.8
X			
Snyder, Rutz and Marion (1955)	Fresh, at 5°C.	3,990	64.0 (a)
	Deep frozen, stored 10 - 140 days at -79°C.	904	67.0
	Deep frozen, stored 122 days at -79°C.	221	58.0
Larson and Graham (1958)	Deep frozen, stored at -79°C.	463	69.3 (b)
	Deep frozen to -79°C. stored at -196°C. in liquid nitrogen	495	71.9

Ref. (1)	Semen storage conditions	First Inseminations	Non-Return Conception Rate (2)
Pickett, Jones, Heller, Cowan and Gosslee (1959)	Fresh, at 5°C.	332	77.7 (a)
	Deep frozen and transferred to liquid nitrogen at -196°C.	329	72.9

Note:- (1) All split sample trials except:-

Y - results obtained over 3 years and not from split samples

X - fresh and frozen results obtained from different samples

(2) Conception rates assessed after the following periods:-

(a) 60 - 90 days (b) 75 days (c) 90 days (d) 112 days (e) 90 - 120 days

S E C T I O N X

INSEMINATION TECHNIQUES : TIME OF INSEMINATION, HEAT DETECTION AND OTHER MANAGEMENT FACTORS INFLUENCING CONCEPTION RATES

A. Insemination techniques

The original method using a vaginal speculum and the later method using the rectal technique were described by Anderson (1945). Since then, the rectal method (which was reported by Salisbury and Van Demark (1951) to have been first introduced into the U.S.A. from Denmark in 1938 by Larson) has been almost universally adopted. This gave favourable results when used under field conditions (Rowson, 1944; Lasley and Bogart, 1943) for a small number of inseminations. Holt (1946) found that the intra-uterine deposition of the semen by the rectal technique was superior to the intra-cervical insemination with a speculum, but different operators were used for each technique. The rectal method gave also higher conception rates than the speculum technique in the work reported by Raps (1948), who used an intra-cervical site for semen deposition with both techniques; Hendrickse and Van Der Kaay (1950), using large numbers of cows, confirmed the superiority under practical conditions of the rectal technique, Although these workers compared two different techniques for introducing the semen into the genital tract, the actual site of deposition of the semen also differed. With the speculum technique, this was shallow or mid-cervical whereas, with the rectal technique, the semen was generally deposited at

the intra-uterine site; this was not possible with the speculum technique (Trimberger, 1942). Whilst there was no evidence that satisfactory conception rates could not be achieved by proper use of the speculum technique with semen, used either fresh or at a very low dilution soon after collection, Weeth and Herman (1951) reported that, with the intra-uterine rectal technique, the conception rates with semen stored 12 or 24 to 36 hours were similar, whereas, with the intra-cervical site of deposition by the speculum method, the 24 to 36 hour old semen had a significantly lower conception rate than that stored for 12 hours. However, when stored semen and possibly also high dilution rates are employed the rectal method is favoured; it ensures the correct deposition of the semen, but the speculum method is still extensively used in Russia (Ozin, Parshutin, Rodin, Skatkin and Sergin (1956).

B. Volume of semen inseminated

The insemination of 1 ml. diluted semen by the rectal technique has been generally accepted as satisfactory. Olds, Seath, Carpenter and Lucas (1953) found no significant difference in the conception rates obtained with either 0.25, 0.5, 1.0 or 2 ml. diluted semen, containing 24 million or 16 million spermatozoa/ml. There was a tendency for the larger insemination dose to produce a higher conception rate than the smaller dose, this being more marked when the largest volume, with the highest spermatozoal density, was deposited at the intra-uterine site. Similarly, Lasley and Bogart (1943) found no conception rate

differences following the use of either 0.5 ml. or 1 ml. of semen (either undiluted or at a very low dilution rate). These findings can only be applied to the use of the rectal technique. The effect of insemination volume when using semen at a high dilution rate has not been extensively studied.

C. Types of insemination instruments

The types of instruments used with the rectal technique vary somewhat. The pipette, described by Rowson (1944), is still extensively used. This consists of a glass tube (1 ml. of semen should not fill more than two thirds of its length), a 2 ml. syringe pump, attached by a rubber connector to one end, is used to draw the semen into the lumen and also to expel it at the actual insemination. A clean pipette is used for each insemination. In some countries (U.S.A. and New Zealand) plastic pipettes, which are discarded after each insemination, are now used. With these the risks of injury to the cow following pipette breakage, and of the introduction of disease, are obviated. However, the chief advantage would seem to be the savings on the costs of cleaning, sterilising and preparation since, out of 3,568,624 inseminations, only 14 cases of uterine injury due to the glass pipette were reported by the Milk Marketing Board (Report, 1957). This report claimed also that operators, accustomed to the use of rigid glass pipettes obtained lowered conception rates with the plastic ones; their conclusions were, however, based on a small scale trial in which glass and plastic pipettes were used on alternate days, the operators having a limited chance to accustom themselves to the new technique.

At the Reading Cattle Breeding Centre, (Report, 1958), conception rates of 62 per cent and 60.8 per cent were obtained with glass and plastic pipettes, respectively, over a total of 9,507 first inseminations when the operators used glass and plastic pipettes over alternate 2 week periods. Romanowski (1957) claimed an improved conception rate with plastic pipettes, but this was based on smaller numbers of inseminations and this observation was not strictly controlled.

In Denmark, Sorensen (1946) described an insemination instrument for use with the rectal technique. Semen was contained in a cellophane tube which fits into the detachable end of the metal pipette, running the complete length of which was a metal rod, which, when pushed through the cellophane tube, acted like the plunger in a syringe and deposited the semen at the required site. The complete apparatus was carried between farms in an alcohol bath to sterilise it, and a separate and previously sterilised end piece was used for each cow. Being unbreakable this instrument will last indefinitely - but it must be sterilised between farms. A similar method of introducing the semen has been adopted by Cassou (1950), who reported the storage of semen in special straw-like containers, sealed at both ends with an alcohol powder which formed a jelly like plug.

Other less orthodox methods of insemination have been reported by McDonald and Sampson (1957), who obtained one pregnancy in four heifers in which the semen was injected intra-peritoneally through the flank; Skjervøen (1955) obtained conception in a dairy heifer by introducing semen into the abdominal cavity by passing a cannula through the vaginal

wall near the fornix. Fechheimer, Ludwick and Ely (1952) carried out intra-uterine inseminations by direct injection into the uterine horns per rectum, three of the four cows inseminated by this method became pregnant. Whilst these methods could not be adopted routinely they may be of use when a particularly valuable animal cannot be inseminated by the normal methods as, for example, in a case of cervical occlusion. This could only be carried out by an operator fully conversant with the anatomy of the genital tract and of the related abdominal organs. Field observations on the occurrence of cervical occlusion were made by Flerchinger, Erb and Ehlers (1956,a), who reported a failure to pass the insemination tube through the cervix in 4 per cent of the cows and 40 per cent of the first service heifers, with a resultant lowered conception rate. However, Olds and Seath (1954) were unable to penetrate the cervix in 1.1 per cent of 11,112 cows and 11.7 per cent of 1,711 heifers; the conception rates in these animals following, presumably, an intra-cervical insemination were 36.5 per cent and 54.0 per cent respectively. The authors presumed that in the heifers this constriction was not due to an abnormality whereas in the cows this condition was considered to be associated with an abnormal state of the cervix and hence they had a lowered conception rate. Munro (1956) reported that cervical constriction occurred in 0.1 per cent of 16,238 animals presented for insemination. Following the injection of dienoestrol, dilation of the cervix occurred in 11 out of 15 animals so treated. A simple method of insemination was described by Maruskin and Sivókonj (1939), who introduced into the cervix, by hand per vagina, a gelatine capsule containing semen at a very

low dilution (500 to 150 million spermatozoa were used per insemination). This technique, which could be used by unskilled operators, has, however, not been reported on extensively, and it could only be expected to give a reasonable level of fertility with semen used either undiluted or at a very low dilution rate. Davis, Underbjerg and Trimberger (1940,b) described a similar method in which a 2 ml. gelatine capsule of semen was deposited into the anterior vagina by means of an "insemination gun". In a limited number of cows (170), the conception rates were only slightly lower than those obtained with the normal cervical method, and it was claimed that this could be used by unskilled personnel. However, since this technique requires the use of semen at a low dilution rate, its usefulness in the field would appear to be limited, since a large number of bulls would be required in order to get sufficient semen for a normal service.

D. Site of semen deposition, effect on fertility

The average period of survival of the spermatozoa in the genital tract was reported by Trimberger and Davis (1943) and by Laing (1945,b) to be about 30 hours, and by Vandeplassche and Paredis (1949) to be as long as 56 hours. However, since it was previously thought that the spermatozoa required several hours to ascend to the Fallopian tube, it was considered preferable to deposit the semen intra-uterine, as close as possible to the site of fertilisation (Brewster, May and Cole, 1940). Later, Van Demark and Moeller (1951) indicated that spermatozoa could travel to the ovarian end of the oviduct within 2.5 minutes of

being placed in the cervix; this was considerably faster than previously believed possible and, therefore, the use of an intra-cervical site of semen deposition was considered. Moreover, the widespread use of artificial insemination focussed attention on the risks of producing abortion after the accidental insemination of a pregnant cow. The incidence of oestrus in pregnant cows appears to vary somewhat; it was reported as being 3.4 per cent by Donald (1943) 4.5 per cent by Rahlman and Mead (1958) and 6 per cent by Donoho and Rickard (1955). There appears to be a tendency for this to occur in the first three months of pregnancy. In view of the foregoing, several workers carried out fertility trials with different sites of semen deposition using the rectal techniques (see table 13). Salisbury and Van Demark (1951) obtained conception rates of 64.0 per cent, 65.1 per cent and 64.8 per cent with over 2,000 inseminations at the cervical, uterine body, and uterine horn sites, respectively; these results were in accordance with the report of Stewart and Melrose (1952) who obtained conception rates of 64.5 per cent and 64.6 per cent at the cervical and uterine body sites, respectively, with a total of 8,833 inseminations. However, Knight, Patrick, Anderson and Branton (1951) obtained a significantly higher conception rate with inseminations at the uterine body site than at the uterine horn, or cervical, or cervical plus uterine body sites; similarly Olds et al. (1953) demonstrated, in a trial involving 9,558 inseminations, that the simultaneous deposition of semen at all three sites, (i.e. cervical, uterine body and uterine horn) tended to give the highest conception

rate, although such a technique could not always be adopted for routine field work. More recently Tjupic (1957), in a study limited to 48 cows, found that spermatozoa remained motile for 16 hours after intra-uterine and for 25 hours after ~~the~~ intra-cervical insemination. This was taken to indicate that the cervix prevented the transport of dead spermatozoa into the uterus; on a limited trial, involving a total of 196 cows, 68 per cent conceived to intra-uterine insemination and 79 per cent to intra-cervical insemination.

By the deliberate intra-uterine insemination of cows at 40 to 150 days of pregnancy, Van Demark, Salisbury and Boley (1952), Stewart and Melrose (1952) and Tanabe, Heist and Almquist (1955) confirmed that there was a great risk of inducing abortion with the intra-uterine technique. Van Demark *et al.* (1952) found that the inclusion of anti-biotics in the diluent reduced the risks of abortion and Tanabe *et al.* (1955), in confirmation of these findings, claimed that the abortion was due to the setting up of a bacterial infection and not to the actual mechanical damage from the introduction of the insemination catheter. Pounden, Ferguson, Knoop and Krauss (1947) have suggested that intra-uterine insemination could result in an endometritis. Rowson, Lamming and Fry (1953) have demonstrated that this can arise following interference with the uterus in the luteal phase of the oestrous cycle, as could accidentally occur in routine insemination work. Manthei, De Tray and Goode (1950) claimed that a non-motile organism, like *Brucella abortus*, would not readily become established in the uterus following

its deposition in the intra-cervical site.

Although, with one exception, the above studies indicated a tendency for a higher conception rate with insemination at the intra-uterine site, there are, as indicated above, definite risks associated with this method and they can be avoided by using the deep cervical technique. The actual site of insemination probably varies with different centres and also between operators. Since there is a danger of an inexperienced operator only using a middle or even a shallow cervical site instead of a deep cervical site, it would appear adviseable to train all inseminators to use the intra-uterine technique initially, and subsequently to change them over to the deep cervical method.

E. Factors influencing the effectiveness of the insemination in the field

(i) Heat detection. Since artificial insemination operators have to rely on the observations of the stockmen, the latter must be encouraged to plan the management of the herd in order to obtain the highest degree of efficiency in the detection of oestrus. In the short hours of daylight during the winter, particular attention must be paid to checking the animals for heat in the morning and again in the afternoon; this can only be done when the animals are at rest and not primarily interested in feeding. Negative reports on objective methods of detecting heat have been made by Van Demark and Estergren (1959), who used ultraviolet light to detect fluorescence in the vulva, and by Alliston, Patterson and Ulberg (1958), who obtained negative results

in a study of the crystallisation pattern of cervical mucus. Scott Blair, Folley, Malpress and Coppen (1941) observed that the flow-elasticity of cervical mucus showed a definite maximum at or about the time of oestrus, and devised a simple instrument, the 'oestroscope', to measure flow-elasticity with the intention of providing the farmer with an objective method of detecting oestrus. However, the method has not come into general use and, in an experiment to assess the practical value of the oestroscope, Blackburn and Castle (1959) concluded that it was unlikely to be used because of the wide variations in the maxima and of the uncertainty of detecting the precise day of oestrus. Similar variations in the flow-elasticity had been reported previously by Roark and Herman (1950). Simunic (1954) could find no relationship between the appearance of the oestrous mucus at the time of insemination and conception rate, and Rottensten and Touchberry (1957), from a study of the oestrous behaviour of heifers at progeny test stations, concluded that there would be practically no gain in conception rate by selecting for the degree of expression of heat. Trimberger and Davis (1943) and Dyrendahl (1945) indicated that more than 50 per cent of the animals came into heat overnight or in the early morning. The necessity for adopting a herd management routine, which allows the maximum chance of detecting oestrus, was emphasised by Bearden (1957), who reported a 60-90 day non-return conception rate of 64.1 per cent for cows not turned out for heat detection during the winter; whereas, with cows turned out once or twice daily the conception rates were 69.5 and 70.4 per cent, respectively. In this

study the average conception rate in herds of 10 to 29 cows was 70.3 compared with 65.6 per cent in herds of 60 cows or more. The behaviour, or rather the change in behaviour, of the animal in oestrus must therefore continue to be the only reliable indicator for the stock owner.

(ii) Timing of insemination, hormonal control of and duration of oestrus. The optimal period during which a satisfactory conception rate can be expected following the onset of heat is of considerable importance for the running of an insemination centre. Barrett and Casida (1946), using data recorded by owners on the exact time 3,841 cows were first noticed in oestrus, found there was little variation in conception rate with cows bred at 3 to 25 hours after being noticed in heat. Trimberger (1948) used experimental animals, which were checked for heat every two hours, and concluded that the best conception rates were obtained with inseminations not less than 6 and not more than 24 hours before ovulation, i.e. approximately 4 to 22 hours after the onset of heat. Extensive field observations on the period after the onset of heat, during which insemination will prove satisfactory, have confirmed that, with insemination done up to 24 hours after the onset of heat, a satisfactory conception rate should result. In addition, a reduction in fertility with inseminations carried out within 4 to 6 hours of the onset of heat was indicated, but opinions varied as to the optimum period for highest fertility (Valerani, 1950;, Schindler and Volcani, 1952; Patrick and Herman, 1953; Uray, 1955; and Bonfert, 1956,b). Although Larson and Bayley (1955) and Autrup and Rasbech (1951), in a study of inseminations

carried out in cows showing a post-oestrus haemorrhage, confirmed that conception could result in about 25 per cent of cows inseminated 48 hours after the end of the oestrus, they suggested that these cows were abnormal since there may have been a delayed ovulation. Although it has not yet been demonstrated that bovine spermatozoa require to undergo a period of maturation or "capacitation", as was shown to be necessary in the rabbit (Chang, 1951), if this was proved to occur also in the bovine then insemination close to the time of ovulation would be contra-indicated. It must be pointed out that with studies under field conditions the animals would normally be checked only twice daily for oestrus, and thus the time intervals quoted would not be exact; therefore, reference is made only to studies involving 1,000 or more animals. The recommendations made originally by Trimberger (1948), as a result of the two hourly observations, have been proved correct by reports from the field, i.e. cows, seen in season for the first time in the morning up to 10 o'clock, should be inseminated on that day, whilst those coming into heat later on that day should be inseminated on the following morning. It must be emphasised that these recommendations were based on the prompt detection of the onset of heat. In practice, in view of the inevitable delay between the cow being seen in season and the inseminator arriving at the farm, there is little chance of the insemination being done too early in the heat. If the use of a nominated service requires the insemination request to be made to the centre earlier than usual, such owners must pay strict attention to this prompt detection of heat, otherwise a reduced conception rate could

result from delay in insemination. Dyrendahl (1945) studied the effect on the overall conception rate of suspending inseminations on Sundays; it was estimated that the reduced chances of conception in cows, in season on Sunday and held over to Monday, would mean a drop of 8 per cent in the centre's overall conception rate; although such an arrangement would considerably facilitate the running of a centre, it could result in a lowered conception rate, but this could possibly be partly reduced by meeting all requests received up to a late hour on the Saturday afternoon. Schindler, Volcani and Angel (1957) found that when no service was given (i.e. on Sunday and on holidays) on the subsequent day the conception rate for 4,430 inseminations was 52.1 per cent, whereas, the overall conception rate was 55.7 per cent with 17,492 inseminations carried out over an eleven month period. These authors found that 25 per cent of these post-holiday inseminations were done later than normal, and in this delayed group the conception rate was only 42.7 per cent.

(iii) Two or more inseminations during one oestrus. Requests for double insemination within one heat period cannot be economically met in commercial artificial insemination. Trimberger and Davis (1943), Jondet (1955) and Ivankov (1959) have reported an improved conception rate with this. However, Aschbacher, Smith and Stone, (1956) and Wilcox and Pfau (1958) have reported no beneficial effect, even when three inseminations were given within one heat period. It would appear that this practice could only be of benefit in cows with delayed ovulation, and a proper assessment of its value could only be

made with such cases.

(iv) Effect of insemination on the cow. Hays and Van Demark (1953); Van Demark and Hays (1955) investigated the release of oxytocin and its direct effect on spermatozoal transport by the induction of uterine muscle contractions. This work suggested that any conditions likely to lead to the release of adrenalin within the cow, such as insemination of a nervous cow or faulty insemination technique, might inhibit the travel of the spermatozoa within the genital tract. A study, by Pounden and Firebaugh (1956), of the degree of nervousness shown by 1,445 cows (2,009 inseminations), whilst not confirming this, did indicate that a lowered conception rate might be obtained with either completely quiet or highly nervous cows, but the results were inconclusive. In a subsequent report, Hays, Van Demark and Ormiston (1958) indicated an improved conception rate both to insemination and natural service in a limited number of cows after oxytocin injections, but there was no indication of lowered fertility after adrenalin injection; the dose of the latter employed was subsequently found to be inadequate, and further investigation would appear necessary to decide whether or not insemination is contra-indicated in a nervous cow.

(v) Optimum time for insemination after calving. Although on clinical examination the uterus of the healthy cow appears to have involuted and returned to its normal size by about the 26th day post-partum (Casida and Venzke, 1936), there is now extensive evidence to

show that for optimum fertility a much longer interval between parturition and insemination is necessary. Edwards (1950) found that fertility increased when inseminations were done up to 90 days post-partum and levelled out thereafter. Although Shannon, Salisbury and Van Demark (1952), using insemination, and Trimberger (1954), using natural service, indicated that a lower minimum interval (51-60 days) should be allowed, most other reports confirm that at least 60 days are required. The conception rates following natural service, studied by Van Demark and Salisbury, (1950), Beshlebnov (1956), Bower and Merilan (1958), were in accord with this, since they found that 60-80 days were required, but Olds and Seath (1954) and Søgnen and Filseth (1958) found the highest conception rates were obtained 2-4 months post-partum. Since this factor is dependent on the state of health of the uterus, a much longer interval would be desirable if any disease condition had been present. From the available evidence, a general recommendation of a minimum interval of 60 days could be made. It must be emphasised that Trimberger (1954) found no appreciable effect of the interval between parturition and first service on the incidence of retained placenta, metritis or abortion or on subsequent conception. Apart from the adverse effect on the herd conception rate, there is no evidence of any adverse effect on the individual animal following a reduction of this interval.

F. Efficiency of the inseminator. In most centres the conception rates are assessed for operators as well as for bulls and there have been few references in the literature to the operator

conception rate variations. Although with fully experienced operators the numbers of inseminations, i.e., the pressure of work, can be taken to have little effect on the conception rate, this can only apply within certain limits, and the amount of possible work will also depend on the terrain of the area the operator covers. Since the costs of salaries and travelling have been shown (Milk Marketing Board, Report, 1959,a) to account for 55.7 per cent of the costs of the insemination service, it is most important that the most efficient use is made of the inseminators' time i.e. by getting the maximum conception rate. With a group of 12 experienced operators, carrying out 5,554 first inseminations with the intra-uterine technique, Stewart and Melrose (1952) reported an overall 112 day non-return conception rate of 64.6 per cent, with the individual operator conception rates varying from 60.2 to 69.8 per cent. There is little published information on the normal variation in results obtained by an experienced operator over a period of, for example, one year, but Smith (1956) has shown how the overall 90-120 day non-return conception rates of 29 newly appointed technicians improved from 50.7 per cent for their first month to 61.3 per cent for their third month in the field; a study of the individual operator's results showed, however, that in some cases up to six months were required before the expected conception rate was recorded. Although the individual inseminator's technique may effect the release of oxytocin and, therefore, the rate of travel of the spermatozoon in-utero, this has not been shown to account for individual

operator variations. Tjupic (1957), in a limited but controlled observation, obtained a conception rate of 78 per cent with 50 cows, in which the uteri were massaged for 1-2 minutes prior to insemination, and 59 per cent with 80 untreated control cows. Although the speed with which the insemination is carried out has not been shown to bear any relation to conception rate, the effect of the handling of the uteri could possibly be studied further.

G. Ovulation delay and hormonal factors controlling it. Although ovulation occurs spontaneously in the cow, recent research by Armstrong and Hansel (1959) has shown that it can be induced by the action of a secretion from the hypothalamus on the anterior pituitary and, therefore, drugs acting on the hypothamamus might prove useful in the control of ovulation. Asdell (1958) has posed the question as to whether or not the spermatozoa from high fertility bulls fertilise more eggs that do those from low fertility bulls, because spermatozoa from the former can survive longer in the female tract and are not so likely to be ineffective on account of delayed ovulation. The studies of Marion, Smith, Wiley and Barrett (1950), on the effect of sterile copulation on ovulation time in maiden heifers, suggested that the act of service may have some effect on the mechanism for inducing ovulation, but the effect of insemination on ovulation time has not been studied. Relating the time of insemination to the time of ovulation rather than to the time of heat onset may markedly affect fertility.

Trimberger (1956) indicated that the breeding of cows with irregular cycles should not be restricted; whilst a refusal to inseminate such cows would undoubtedly help an insemination centre to maintain a satisfactory overall conception, there is some justification in inseminating them as there is a chance, even although it is reduced, of conception in such cases.

In general, therefore, unless the users of insemination can ensure the prompt detection and reporting of oestrus a lowered conception rate can result through faulty timing of the insemination. The efficiency of the operator is also of considerable importance; although faults in the insemination technique can be reflected in a lowered conception rate, very often the reason for a reduced fertility with individual operators is not readily obvious.

TABLE 13

COMPARISON OF INSEMINATION TECHNIQUES

Ref.	Method and Site of Semen Deposition	Number of First Inseminations	Non-Return Conception Rate Per cent
Holt (1946)	Speculum - intra-cervical	377	33.4
	Rectal - intra-uterine	374	56.1
Raps (1948)	Speculum - intra-cervical	931	53
	Rectal - deep cervical	789	61
Hendrikse and Van Der Kaay (1950)	Speculum - intra-cervical	1,789	48.3
	Rectal - intra-uterine	2,708	60.6
Knight, Patrick Anderson and Branton (1951)	Rectal - Uterine and Cervical	503	62.4
	Rectal - Cervix	500	58.4
	Rectal - Uterine body	500	64.8
	Rectal - Uterine horns	511	62.6
Salisbury and Van Demark (1951)	Rectal - deep cervical	2,327	64.0
	Rectal - Uterine body	2,122	65.1
	Rectal - Uterine horns	2,151	64.8
Stewart and Melrose (1952)	Rectal - Mid-cervical	4,279	64.5
	Rectal - Uterine body	4,554	64.6
Olds, Seath, Carpenter and Lucas, (1953)	Rectal - Cervix	*	66.2
	Rectal - Uterine body	*	65.7
	Rectal - Uterine horns	*	67.9
	Rectal - All three sites	*	69.0

* Number of inseminations done at each site ranged from 174 to 435; average = 298.7

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